factor may tether it to the enzyme and allow access of the site to nucleotide substrate and release of product. The bifunctional DHFR-TS from *Leishmania major* has an unusual charge distribution that could account for channelling of folate cofactors between active sites *(55)*. Human TS has been cloned, sequenced, expressed *(56,57)*, and crystallized *(58)*. The crystal structure suggests a mechanism for docking of substrates involving the pivoting of an active site loop.

Purified cellular human and rat thymidylate synthases are acetylated at their N-terminal amino acids and have a lower specific activity than the corresponding recombinant enzymes *(57)*. In H35 rat hepatoma cells, TS has been localized to the nucleolar region but appears in the cytoplasm when overexpressed *(59)*. TS was also present in the mitochondria of H35 cells and a small amount of phosphorylated TS was identified. TS binds to its own mRNA as a negative regulator *(60)*. This binding requires that TS be blocked at its N-terminal position. Elements in the promoter region of the human TS gene have been identified and the nuclear factor Sp1 is a major contributor to promoter activity, but other positive and negative regulators have been identified *(61)*. Further studies of these modifications and interactions should elucidate the relationship of thymidylate synthase to the cell division cycle.

Accumulation of dUTP and its misincorporation into DNA is a major factor in the cytotoxicity resulting from the inhibition of TS. dUTPase catalyzes the conversion of dUTP to dUMP and therefore acts to counteract the toxic action of dUTP *(62)*. Conversely, inhibitors of dUTPase should enhance the toxicity of TS inhibitors. X-ray crystallographic studies show that the active site of human dUTPase, a trimeric enzyme, consists of residues from all three subunits *(63)*. The human dUTPase gene codes for both nuclear and mitochondrial isoforms of the enzyme *(62)*.

3.4 Dihydrofolate Reductase

In contrast with SHMT (a tetramer) and TS (a dimer), human DHFR is monomeric (22 kDa). It catalyzes the reduction of 7,8-H_2PteGlu$_n$ to 5,6,7,8-H_4PteGlu$_n$ *(64)*. The human enzyme has been cloned, expressed, and crystallized *(65)* and the 1H and 15N nuclear magnetic resonance assignments obtained *(66)*. Again in contrast with SHMT and TS, the primary structures of eukaryotic DHFRs are not highly homologous, only 20% of the residues of human DHFR are identical to those found in eight other eukaryotic DHFRs *(64)*.

Site-directed mutagenesis studies have led to the production of variants of human DHFR resistant to methotrexate *(67,68)*. Current studies in mice are testing the concept that cDNA coding for methotrexate-resistant DHFR transduced into bone marrow progenitor cells will lead to improved curability of mice bearing a methotrexate-sensitive tumor *(67,68)*.

Folate polyglutamates and antifolate polyglutamates often have a modest two- to 10-fold enhanced affinity for human DHFR as compared with monoglutamate forms *(64,69)*. An interesting exception is 2-desamino-2-methylaminopterin, which has an IC_{50} value greater than 50 μM but the addition of four γ-linked glutamyl residues lowers the IC_{50} >200-fold to 0.25 μM *(70)*.

The cellular synthesis of human DHFR is negatively regulated by the binding of the enzyme to its cognate mRNA *(71,72)*. Methotrexate binding to DHFR prevents this interaction and promotes DHFR production.

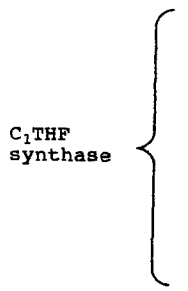

Fig. 6. Interconversion of

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and S for dehydrogena
functional enzyme has
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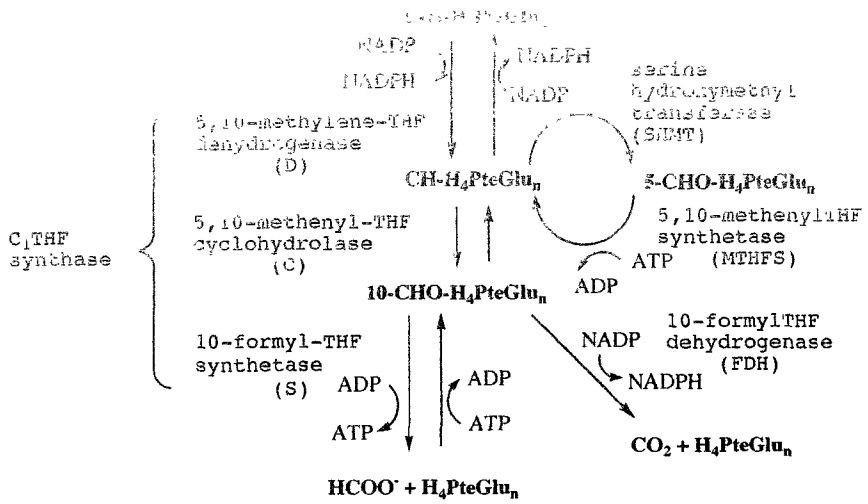

Fig. 6. *Interconversion of methylene and formyl tetrahydrofolate derivatives.*

The activity of the murine DHFR gene promoter increases at the G1-S-phase boundary of the cell cycle, mediated by a member of the E2F family of transcription factors *(73)*.

4. C1-THF-SYNTHASE

C1-THF-synthase is a homodimeric enzyme complex that occurs in two forms in mammalian cells *(74)*. One form is a trifunctional, cytoplasmic, 100 kDa, $NADP^+$-dependent 5,10-CH_2-$H_4PteGlu_n$ dehydrogenase-5,10-CH-$H_4PteGlu_n$ cyclohydrolase-10-CHO-$H_4PteGlu_n$ synthetase (Fig. 6). The three enzyme activities are abbreviated D, C, and S for dehydrogenase, cyclohydrolase, and synthetase, respectively. The human trifunctional enzyme has been cloned, sequenced, and expressed *(75)*. The amino-terminal 34-kDa DC portion of the trifunctional enzyme has been expressed separately and is convenient for kinetic studies. The trifunctional cytoplasmic DCS complex is most likely necessary to catalyze the incorporation of formate, arising from serine, glycine, and methyl groups in mitochondria, into 10-CHO-$H_4PteGlu_n$ for use in purine nucleotide synthesis and into 5,10-CH_2-$H_4PteGlu_n$ for use in dTMP and methionine synthesis *(76–78)*. This role for the trifunctional enzyme is supported by kinetic and metabolic studies.

The second form is bifunctional NAD^+-dependent DC, which is a nuclear-encoded 34-kDa mitochondrial enzyme. The human and murine bifunctional mitochondrial enzymes have been cloned, sequenced, and expressed *(79)*. This system has been suggested to serve as the source of formyl groups for the synthesis of formylmethionyl tRNA required for protein synthesis in mitochondria. Its location in mitochondria is consistent with its resemblence to DC enzyme complex found in bacteria *(79)*. In contrast to the DC portion of the cytosolic trifunctional enzyme complex, it has an absolute requirement for Mg^{2+} and P_i *(80)*. It is proposed that Mg^{2+} and P_i substitute for the 2′ phosphate on the adenosine portion of NADPH because it has a 44% amino acid sequence identity with the DC domain of yeast mitochondrial NADP-dependent trifunc-

tional enzyme, the human NAD-dependent enzyme has a low, Mg^{2+}-dependent turnover with NADP, and P_i competes for NADP binding.

Kinetic studies provide an explanation of the mechanism by which the mitochondrial NAD-dependent DC enzyme functions to convert 5,10-CH_2-H_4PteGlu$_n$ to 10-CHO-H_4PteGlu$_n$, whereas the cytosolic NADP-dependent enzyme functions in the reverse direction *(78)*. In the cytosolic system, where the NADPH/NADP ratio is high, the rate-limiting C-reaction is stimulated by NADPH analogs, and presumably by NADPH as well, but technical difficulties prevent a direct test. The 10-CHO-H_4PteGlu$_n$ is 100% channeled for reduction to 5,10-CH_2-H_4PteGlu. In mitochondria, the NADH/NAD ratio is low, favoring the oxidative reaction and the conversion of 5,10-CH_2-H_4PteGlu$_n$ to 10-CHO-H_4PteGlu$_n$ which is not stimulated by nucleotides. Both cytosolic and mitochondrial DC activities are carried out at a single active site.

In contrast with cytosolic trifunctional NADP-dependent DCS, where high activity is found widely distributed among various tissues, the NAD-dependent system is usually not detectable in most tissues of adult animals, but its cognate mRNA is detectable *(81)*. NAD-dependent DC activity is found in embryonic tissue and in most all transformed cultured cells.

The affinity of the DC complexes for folate substrates is not greatly enhanced by increasing the polyglutamate chain length. Monoglutamate forms function in substrate channeling as well as polyglutamates. This contrasts with the formiminotetrahydrofolate transferase-formiminotetrahydrofolate cyclodeaminase system involved in histidine catabolism, in which affinity and channeling are enhanced with polyglutamate substrates.

The 10-CHO-H_4PteGlu$_n$ synthetase domain of cytoplasmic DCS catalyzes the formation of 10-CHO-H_4PteGlu$_n$ from formate and H_4PteGlu$_n$ accompanied by the hydrolysis of MgATP to MgADP. In contrast with DC activities, S has a very high affinity for its polyglutamate substrate. The K_m value for H_4PteGlu$_5$ for the rabbit liver enzyme is 0.1 μM and the binding of H_4PteGlu$_n$ and MgATP enhance the binding of formate. The activity of S from bacteria and mammals is stimulated by K^+ or by NH_4^+, but Na^+ and Li^+ have no effect *(82)*. Spermine stimulates S from *Lactobacillus arabinosus* and *L. casei* by lowering the K_m of H_4PteGlu$_1$ *(83)*. This observation is noteworthy because spermine reduces the amount of thymidine required to reverse the inhibition of growth of *L. arabinosus* by aminopterin and other antifolates *(84)*. Therefore, spermine might play a regulatory role at the formyl level as well as at the methyl level mentioned above. Both of these stimulations could benefit growing cells by stimulating the formation of single-carbon units for methionine, thymidylate, and purine nucleotide synthesis.

In vitro kinetic studies with rabbit liver DCS coupled to SHMT suggest that the two proteins interact to facilitate the conversion of formate to serine *(76)*. DCS plus SHMT provide an estimated in vivo concentration of 25 μM folate active sites, which indicates that most of the folate coenzymes in cells are protein bound.

[illegible]

COOH-terminal domain (300 amino acids) that resembles aldehyde dehydrogenase, and a connecting peptide of 100 amino acids. It is likely that the major function of FDH is to catalyze the oxidation of the formyl group of 10-CHO-H_4PteGlu$_n$ to CO_2 using NADP as the hydrogen acceptor. This would provide egress of single-carbon units from the cellular one-carbon pool as well as providing NADPH for reductive reactions (Fig. 6). This function requires the appropriate juxtaposition of the NH_2-terminal and COOH-terminal domains provided by the connecting peptide. In addition, the NH_2-terminal domain catalyzes the hydrolysis of 10-CHO-H_4PteGlu$_n$ to H_4PteGlu$_n$ and formate. This activity, combined with that of C1THF synthase, leads to a futile cycle for the production and reincorporation of formate. The isolated COOH-terminal domain has aldehyde dehydrogenase activity and serves this function in the conversion of 10-CHO-H_4PteGlu$_n$ to CO_2 and H_4PteGlu$_n$ by FDH, the 10-CHO group corresponding to the aldehyde portion of formic acid.

FDH has a very high affinity (K_d = 20 n*M*) for both 10-CHO-H_4PteGlu$_5$ and H_4PteGlu$_5$, the latter being the product of both the dehydrogenase and hydrolase activities and a likely product inhibitor *(88)*. Both SHMT and C1THF synthase discharge H_4PteGlu$_5$ from FDH, thus FDH could serve as a distribution point for 10-CHO-H_4PteGlu$_5$ and H_4PteGlu$_5$.

In rat and rabbit liver FDH accounts for 1.2% of the soluble protein. This is equivalent to a concentration of FDH subunits of 42 μM in vivo *(88)*. Adding this to the concentration of folate-binding sites provided by SHMT plus C1THF synthase (26 μM) yields 68 μM. This reinforces the suggestion that most all the folate coenzymes, whose concentration in rabbit liver is estimated at 26 μM, are enzyme bound in vivo especially since the estimate of 68 μM for folate-binding sites does not include other folate enzymes or GNMT.

Since the N-terminal portion of FDH shows homology with GARFT, the GARFT inhibitors DDATHF (5,10-dideazatetrahydrofolate) and 5, DACTHF (a folate analog lacking the tetrahydropyrazine ring) were tested as inhibitors of rat liver FDH *(89)*. DDATHF showed an IC_{50} of 48 μM but 5-DACTHF showed no inhibition at 340 μM. Polyglutamate derivatives were not tested, but this work opens the possibility of influencing FDH activity, and thus one-carbon metabolism, with folate analogs. In this connection it is of interest to consider mice that are totally lacking FDH *(90)*. Although these mice are able to grow and reproduce, their breeding time is greatly extended. The liver folates of these animals were compared with those of normal mice and 10-CHO-H_4PteGlu went from 2.8 nmol/g in normal mice to 7.3 nmol/g in the FDH-deficient mice, whereas H_4PteGlu went from 19.0 nmol/g in normals to 4.4 nmol/g in the deficient strain. Levels of 5-CHO-H_4PteGlu and 5-CH_3-H_4PteGlu were unchanged. These results are compatible with the loss of FDH in that the increase in 10-CHO-H_4PteGlu could be because of diminished ability to metabolize the CHO group and the decrease in H_4PteGlu could be caused by the loss of a major liver H_4PteGlu-binding protein.

6. 5,10-METHENYLTETRAHYDROFOLATE SYNTHETASE

5,10-methenyltetrahydrofolate synthetase (MTHFS) (formerly 5-formyltetrahydrofolate cyclodehydrase) catalyzes the irreversible MgATP-dependent conversion of 5-CHO-H_4PteGlu$_n$ to 5,10-CH-H_4PteGlu$_n$ *(91)*. As discussed above, 5-CHO-H_4PteGlu$_n$ is formed from 5,10-CH-H_4PteGlu$_n$ in a reaction catalyzed by SHMT. The reactions catalyzed by SHMT and MTHFS therefore constitute a futile cycle (Fig. 6) that is proposed

to regulate cellular levels of 5-CHO-H_4PteGlu$_n$, an inhibitor of SHMT *(47)* and AICARFT *(49)*. MTHFS is the only known enzymatic reaction capable of returning 5-CHO-H_4PteGlu$_n$ to the major pathways of one-carbon metabolism and therefore is a key enzyme in the clinical uses of 5-CHO-H_4PteGlu for prevention of methotrexate toxicity and for enhancing the antitumor activity of fluorouracil.

Human MTHFS has been cloned, sequenced, and expressed *(91,92)*. It is a cytosolic 23-kDa protein with little homology to other folate enzymes except for an SLLP sequence found in most enzymes having 10-CHO-H_4PteGlu$_n$ as a substrate. It is highly homologous to rabbit liver MTHFS which was chemically sequenced earlier *(93)*. Some MTHFS has been found in human mitochondria *(91)*, but none in rabbit liver mitochondria *(92)*, which is surprising because 5-CHO-H_4PteGlu$_n$ is probably formed in vivo by rabbit liver mSHMT and would require a mechanism to re-enter the pool of mitochondrial folate coenzymes. However, folate polyglutamates can leave mitochondria *(4)*, which might replace the need for a mitochondrial MTHFS.

Human cytosolic and mitochondrial MTHFS have similar molecular weights and substrate affinities. Both forms show a much higher affinity for 5-CHO-H_4PteGlu$_5$ than for 5-CHO-H_4PteGlu$_1$, as does the rabbit liver cytosolic enzyme. A cDNA isoform for MTHFS encoding a mitochondrial signal sequence has not been reported.

5-CHO-H_4 homofolate (having an additional methylene group between the 9 and 10 positions of H_4PteGlu) is a competitive inhibitor of MTHFS. The K_i values are 0.1 μM for the rabbit enzyme *(94)* and 1.4 μM for human cytosolic enzyme *(91)*. 5-CHO-H_4 homofolate also behaves as a poor substrate for the reaction. The inhibition of MTHFS by 5-CHO-H_4 homofolate in MCF-7 cells provided important evidence that 5-CHO-H_4PteGlu$_n$ inhibits AICARFT in vivo as well as in vitro *(49)*.

7. GLYCINAMIDE RIBONUCLEOTIDE FORMYLTRANSFERASE (GARFT)

The *de novo* pathway for purine nucleotide biosynthesis consists of 10 enzyme-catalyzed reactions starting from 5-phosphoribosyl-1-pyrophosphate, leading to inosinic acid, the precursor of AMP and GMP *(95)*. Two reactions in this pathway, the third and the ninth, require 10-CHO-H_4PteGlu$_n$ as a formyl donor: glycinamide ribonucleotide formyltransferase and aminoimidazolecarboxamide ribonucleotide formyltransferase (AICARFT) (Fig. 7). The gene for mouse and human GARFT encodes a trifunctional protein of 110 kDa, the GARFT activity residing in the carboxy-terminal 29-kDa portion *(96)*. The other two activities on the trifunctional protein catalyze the second and fifth steps on the purine biosynthetic pathway, synthesis of glycinamide ribonucleotide and aminoimidazole ribonucleotide, respectively. The genes for both mouse and human trifunctional protein have been cloned and expressed and a fully functional 23-kDa human GARFT segment has been expressed as well *(95–97)*. The mouse and human genes are very similar.

10-formyl-5,8-dideazafolate and its polyglutamate derivatives are usually employed as substrates in enzymatic studies because they are more stable than the natural substrate, 10-CHO-H_4PteGlu$_n$. 10-formyl-5,8-dideazaPteGlu$_6$ binds to mouse GARFT 10 times more tightly than the monoglutamate *(98)*. These substrate analogs, their deformylated products, as well as the corresponding derivatives of the inhibitor, 5-10-dideazatetrahydrofolate (DDATHF), all bind to the enzyme very tightly with dissociation constants in

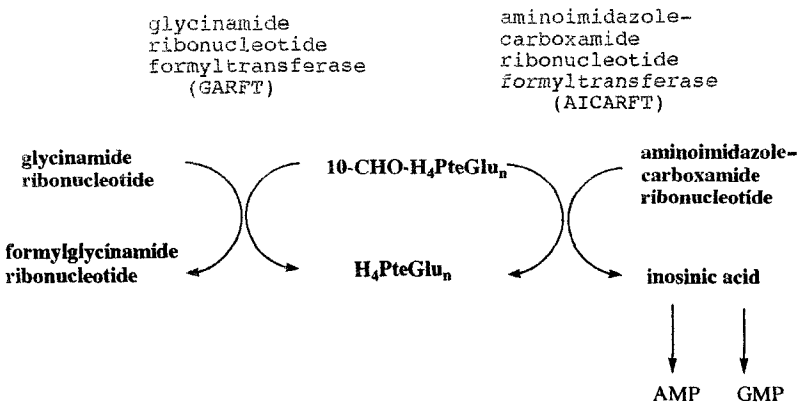

Fig. 7. Folate enzymes involved in purine nucleotide synthesis.

the nanomolar range. Therefore K_i values determined under the standard assay conditions do not reflect true dissocation constants. These studies *(98)* also show that the order of binding of the folate and GAR substrates is random sequential rather than ordered sequential, with the folate substrate binding first as was suggested by studies carried out under the standard conditions *(97)*.

Site-directed mutagenesis studies have identified putative residues for the binding of the polyglutamate chain *(99)*. These residues are located on the opposite lobe of GARFT from that which binds the pteridine portion of the cofactor. The polyglutamate substrate therefore appears to span the active site cleft of the enzyme.

8. AMINOIMIDAZOLECARBOXAMIDE RIBONUCLEOTIDE FORMYLTRANSFERASE (AICARFT)

The ninth and tenth steps on the pathway of the conversion of 5-phospho-ribosyl-1-pyrophosphate to inosinic acid are catalyzed by a bifunctional protein having AICARFT and inosine monophosphate cyclohydrolase (IMPCH) activity, respectively (Fig. 7). The human 64-kDa AICARFT has been cloned, sequenced, and expressed and the two activities have been expressed separately, a 39-kDa carboxy-terminal fragment containing AICARFT activity and a 25-kDa amino-terminal fragment containing IMPCH activity *(100)*. Although both AICARFT and GARFT utilize 10-CHO-H_4PteGlu$_n$ as the formyl donor, there is very little sequence homology between the two enzymes. However, there is a high degree of homology between AICARFT/IMPCH amino acid sequences from different sources.

Polyglutamate forms of coenzymes and inhibitors are more effective with AICARFT than the monoglutamate forms. For example, methotrexate plus four glutamate residues is more than 2000-fold more inhibitory than methotrexate for AICARFT from MCF-7 cells with 10-CHO-H_4PteGlu$_1$ as substrate *(101)*. With 10-CHO-H_4PteGlu$_5$ as substrate, however, the methotrexate polyglutamate was only sixfold more inhibitory than methotrexate. The true K_d values for folate and antifolate polyglutamates with AICARFT have not been determined as they have for GARFT *(98)*. 10-CHO-5,8,10-trideazapteroic acid *(102)* is reported to be an effective inhibitor of human AICARFT *(103)*.

9. METHIONYL $tRNA_f^{met}$ FORMYLTRANSFERASE

In animal mitochondria and in prokaryotes, the initiation of protein synthesis utilizes formyl $tRNA_f^{met}$ *(104)*. The $tRNA_f^{met}$ formyltransferase of animal mitochondria has not been studied extensively. 10-CHO-$H_4PteGlu_n$ is the formyl donor for the reaction for the *E. coli* enzyme which has a strong structural resemblence to *E. coli* GARFT *(105)*. An alternative system to initiate protein synthesis in mammalian mitochondria must be available since cultured cells grow in folate-free RPMI 1640 medium supplemented with thymidine and inosine. The human dietary requirement for folate therefore results from in vivo metabolite deficiencies.

10. FORMIMINOTRANSFERASE-CYCLODEAMINASE

The two activities of this protein serve to catalyze the conversion of the formimino group, arising as formiminoglutamic acid in histidine catabolism, to formimino $H_4PteGlu_n$ and then to 5,10-CH-$H_4PteGlu_n$. The porcine enzyme has been cloned, sequenced, and expressed *(106)*. It is a 480-kDa tetramer of dimers that channels formimino$H_4PteGlu_5$ between the formiminotransferase and cyclodeaminase sites. Both activities require the formation of specific subunit interfaces *(107)*.

11. GLYCINE CLEAVAGE SYSTEM

The glycine-cleavage system is a tetrafunctional enzyme complex found in mitochondria that converts glycine to CO_2, NH_3, and 5,10-CH_2-$H_4PteGlu_n$ *(37,40)*. In the first step, P-protein, a pyridoxal phosphate enzyme, catalyzes the decarboxylation of glycine to CO_2 and an enzyme-bound methylamine group. In the second step, the enzyme-bound methylamine is transfered to lipoic acid (S-S) attached to H-protein. During this transfer, the lipoic acid is reduced to the SH level with the methylamine group still attached. In the third step, T-protein catalyzes the conversion of the attached methylamine to NH_3 and 5,10-CH_2-$H_4PteGlu_n$. The fourth step, the reoxidation of reduced lipoic acid by NAD is catalyzed by L-protein, which is dihydrolipoyl dehydrogenase, an enzyme shared among several mitochondrial α-keto acid dehydrogenases *(108,109)*. The four protein components, P,H,T, and L can be separated from one another by molecular-sieve chromatography.

The glycine cleavage system is the principle route for the catabolism of glycine in mammals and the system is stimulated by glucagon in rat hepatocytes *(110)*. Metabolic leisons in the glycine cleavage system are associated with nonketotic hyperglycinemia, a condition causing severe neurological symptoms in neonates *(111)*. It is suggested that the glycine cleavage system plays a role in regulating glycine levels near N-methyl-D-aspartate (NMDA) receptors in the central nervous system *(112)* that contain a glycine-specific site. Deficiency of the glycine cleavage system leads to increased levels of D-serine in mammalian brain. D-serine occurs naturally in mammalian brain and binds to the glycine site of NMDA receptors *(112)*.

cDNA clones encoding the P, H, T and L components of the human glycine cleavage system have been isolated and their primary structures determined *(111)*. Lipoylated H-protein has been expressed in *E. coli* *(108)*.

12. DIMETHYLGLYCINE DEHYDROGENASE AND SARCOSINE DEHYDROGENASE

These two mitochondrial enzymes provide a pathway for the conversion of the methyl groups of choline, betaine, and methionine to 5,10-CH_2-H_4PteGlu$_n$. Both rat liver enzymes contain covalently bound FAD, have kDa values near 100 and, as isolated, contain H_4PteGlu$_5$ *(113)*. Whereas sarcosine dehydrogenase is very specific for sarcosine, dimethylglycine dehydrogenase shows activity with many N-methyl compounds including sarcosine *(113)*. In the absence of H_4PteGlu$_n$ or if the folate site is blocked chemically, both enzymes continue to oxidize methyl groups unabated, yielding free formaldehyde stoichiometrically *(114,115)*. Dimethylglycine dehydrogenase from rat liver and from rabbit liver bind both H_4PteGlu$_1$ and H_4PteGlu$_5$ very tightly with K_d values < 1 μM *(44,115)*. Rat liver dimethylglycine dehydrogenase has been cloned *(116)*. The enzyme is present in highest amounts in liver and kidney, but low levels are found in many tissues *(117)*. FAD spontaneously binds covalently to rat dimethylglycine dehydrogenase and this binding aids in protein folding and mitochondrial import *(118)*. Sarcosinemia is found in mice lacking sacrcosine dehydrogenase *(119)*.

13. FOLYLPOLY-γ-GLUTAMATE SYNTHETASE

Folylpoly-γ-glutamate synthetase (FPGS) catalyzes the MgATP and K^+-dependent attachment of glutamate residues to the γ-position of folates and folate analog *(4)*. Cells lacking this enzyme cannot retain folates after their transport through the cell membrane and therefore cannot grow. FPGS activity in cells controls the level of folate polyglutamates in cells as well as the glutamate-chain length. Most folate enzymes have a higher affinity for polyglutamate forms of folate coenzymes and folate analogs. FPGS is found in the mitochondria and in the cytosol. Mitochondrial folate accumulation and cytosolic folate accumulation require the activity of mFPGS and cFPGS, respectively. However, pteroyltriglutamates synthesized in mitochondria can move to the cytoplasm and function there, whereas the reverse does not occur, indicating a unidirectional flow of mitochondrial folate triglutamates. Cells lacking mFPGS can synthesize thymidylate and purine nucleotides in the cytoplasm but require glycine for growth. Cells lacking cFPGS require thymine and purines for growth (methionine is routinely added to tissue-culture media) because the mitochondrial Glu chain lengths are longer than three and cannot pass into the cytosol. FPGS activity is increased in proliferating tissues and activity as well as mRNA levels increase after mitogen stimulation and decline during differentiation.

The 60-kDa human FPGS has been cloned, sequenced, and expressed. A single gene with an alternative splice site codes for cytosolic and mitochondrial FPGS, the mitochondrial transcript coding for a 42-residue amino-terminal leader sequence *(120–123)*. H_4PteGlu$_n$ and 10-CHO-H_4PteGlu$_n$ are much better substrates than the corresponding PteGlu, 5-CHO-H_4PteGlu, and 5-CH_3-H_4PteGlu derivatives *(121)*. Thus, under conditions in which methionine synthase activity is low, 5-CH_3-H_4PteGlu$_1$ the major circulating form of folate produced in the liver is poorly polyglutamylated and is not retained after entering cells, leading to folate coenzyme deficiency.

Lowered expression of FPGS is associated with resistance to polyglutamylatable antifolates *(124)*.

14. GLUTAMYL HYDROLASE

γ-Glutamyl hydrolase (GH) catalyzes the hydrolytic cleavage of γ-linked polyglutamates *(125)*. A role for GH in regulating the levels of ptereoylpolyglutamates in cells is indicated since cells expressing high levels of this enzyme show resistance to the polyglutamylatable antifolate DDATHF *(126,127)*. The levels of methotrexate polyglutamates in human blast cells in vivo can be related to their sensitivity to treatment with methotrexate *(128)*. The extent of accumulation of methotrexate polyglutamates has been attributed to the relative activities of FPGS and GH *(129)*.

The gene encoding human GH has been cloned, sequenced, and expressed *(130)*. The 35-kDa protein product has four potential asparagine-containing glycoyslation sites and is a glycoprotein when purified from tissues. Human GH shows 74% homology with rat GH. However the two enzymes show a different pattern of polyglutamate products with 4-NH_2-10-CH_3PteGlu$_5$ as a substrate. Human GH behaves like an exopeptidase, yielding a series of products containing from one to four Glu residues, whereas the rat enzyme is an endopeptidase yielding 4-NH_2-10-CH_3PteGlu$_1$ (methotrexate) as the product. GH is found in lysosomes that have a transport system for methotrexate polyglutamates *(131)*. GH is also excreted from tumor cells *(132)*. Prostate-specific membrane antigen has GH activity *(133)*.

15. CONCLUSIONS

Advances in studies of the genes encoding folate enzymes are empowering investigators with knowledge of the expression of these genes in specific tissues, and tumors, during the cell cycle and during development. Further development of mathematical models of folate and antifolate transport and metabolism will aid in predicting the consequences of inhibiting a given enzyme or combination of enzymes. The interaction of folate enzymes with messenger RNA, the phosphorylation of TS, the potential role of polyamines as regulators, mechanisms of antifolate-induced apoptosis, and levels of DNA methylation are examples of exciting phenomena that could aid the understanding of antifolate selectivity. We eventually should be able to address such problems as:

1. Why do the target cells involved in methotrexate treatment of psoriasis or of rheumatoid arthritis not become resistant to methotrexate?
2. What is the metabolic basis of methotrexate selectivity in the treatment of choriocarcinoma?
3. What is the metabolic basis of the effect of diurnal rhythms on antifolate sensitivity?
4. What is the basis of lipophilic, nonpolyglutamylatable antifolate antitumor selectivity?
5. Why is methotrexate toxic to the liver where cells are not dividing?
6. What folate system is particularly sensitive to folate deprivation in the genesis of neural tube defects?
7. How can agents superior to methotrexate be designed based on knowledge of folate and antifolate metabolism, enzymes, and pharmacology?
8. Are there combinations of folates and antifolates that can maintain cytotoxicity and improve selectivity?

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F. M. SIROTNAK
Laboratory for Molecular Therapeutics
Memorial Sloan–Kettering Cancer Center
New York, New York

J. J. BURCHALL
Wellcome Research Laboratories
Burroughs Wellcome Company
Research Triangle Park, North Carolina

W. D. ENSMINGER
Departments of Medicine and Pharmacology
and the Upjohn Center for Clinical Pharmacology
University of Michigan Medical School
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J. A. MONTGOMERY
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Contents

CHAPTER ONE

The Biochemistry of Folates

Roy L. Kisliuk

Department of Biochemistry and Pharmacology
Tufts University School of Medicine
Boston, Massachusetts

FOLATE ANTAGONISTS AS THERAPEUTIC AGENTS
VOLUME I

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I. Introduction

A dietary factor, subsequently identified as folic acid, proved to be effective in curing megaloblastic anemia of pregnant women in India (Wills *et al.*, 1937). Modern folate studies begin. Folates are synthesized by plants, and folic acid was first isolated from spinach (Mitchell *et al.*, 1941). Many bacteria, including *Escherichia coli*, also synthesize folates, but others such as *Lactobacillus casei*, *Streptococcus faecium*, and *Pediococcus cerevisiae* resemble animals in having a nutritional requirement for folates. Microbial and animal models were vital in elucidating the nature and metabolic significance of these substances.

Folate derivatives are coenzymes for the transfer, oxidation, and reduction of single-carbon units used for the biosynthesis of thymidylate, purine nucleotides, choline, serine, glycine, and many other compounds (Blakley, 1969). In addition, folates play a structural role in some bacteriophages (Kozloff *et al.*, 1956) and serve as chemoattractants in slime molds (DeWit *et al.*, 1983) and *Dictyostelium* (DiNallo *et al.*, 1982).

Folate biochemistry has always been closely associated with the development of outstanding chemotherapeutic agents. Sulfonamides block the biosynthesis of folates and selectively inhibit the growth of many microorganisms (Woods, 1940). Methotrexate (Johns and Bertino, 1982) and trimethoprim (Harvey, 1982; Hitchings, 1983) block folate coenzyme metabolism and are useful antitumor and antimicrobial agents, respectively.

The following is a sampling of the many reviews, monographs, and symposium volumes available in this area: Wolstenholme and Cameron (1954), Hoad (1954), Rabinowitz (1960), Huennekens (1968), Blakley (1969), Bertino (1970), Roder and Huennekens (1973), Pfleiderer (1975), Broquist *et al.* (1977), Bonse and Reynolds (1979), Kisliuk and Brown (1979), Kisliuk (1981), Lowe *et al.* (1983), and Blair (1983). This chapter emphasizes developments in the chemistry, enzymology, and metabolism of folate coenzymes.

II. Chemistry

INTRODUCTION

The structure of H_4PteGlu (tetrahydrofolate) is shown in Fig. 1. It consists of a reduced-oxy-6-methylenetetrahydropteridine linked to *p*-aminobenzoic acid,

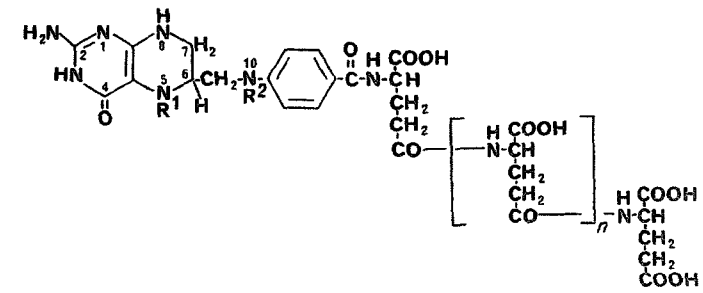

FIG. 1. Structure of tetrahydrofolic acid with γ-linked glutamic acid residues attached.

which is in turn linked to the α-amino group of L-glutamate. The single-carbon units either are carried on the N-5 or N-10 position, or form a bridge between N-5 and N-10 (Table I). These single-carbon units are found at three levels of oxidation corresponding to formic acid, formaldehyde, or methanol.

Derivatives of H_4PteGlu are easily confused because of their similar names

TABLE I

TETRAHYDROFOLATE DERIVATIVES

Name (see Fig. 1 for structure)	R^1	R^2	Abbreviation	Oxidation level of single-carbon unit
5,6,7,8-Tetrahydrofolate	H	H	H_4PteGlu[a]	—
5-Methyl 5,6,7,8-tetrahydrofolate	CH_3	H	5-CH_3-H_4PteGlu	Methanol
5-Formyl 5,6,7,8-tetrahydrofolate[b]	CHO	H	5-CHO-H_4PteGlu	Formate
5-Formimino-5,6,7,8-tetrahydrofolate	HC=NH	H	CHNH-H_4PteGlu	Formate
10-Formyl 5,6,7,8-tetrahydrofolate	H	CHO	10-CHO-H_4PteGlu	Formate
N-5—N-10 Bridge forms				
5,10-Methylene 5,6,7,8-tetrahydrofolate	—CH_2—		CH_2-H_4PteGlu	Formaldehyde
5,10-Methenyl 5,6,7,8-tetrahydrofolate	—CH=		CH-H_4PteGlu	Formate
Oxidized forms				
7,8-Dihydrofolate	$\Delta^{5,6}$		H_2PteGlu	—
Folate	$\Delta^{5,6}$ and $\Delta^{7,8}$		PteGlu	—

[a] Pte, Pteroic acid, or *p*-[(2-amino-4-hydroxy-6-pteridylmethyl)amino]benzoic acid. The total number of glutamate residues is indicated by a subscript; that is, H_4PteGlu$_3$ (Fig. 1) denotes tetrahydropteroyl triglutamate.

[b] Also called folinic acid, leucovorin, citrovorum factor, or CF.

[illegible] methenyl tetrahydrofolate, methylene tetrahydrofolate, and methyl tetrahydrofolate) and because the oxidation level of the single-carbon unit is not always immediately obvious. For example, in 5-CHO-H_4PteGlu the formyl group is at the oxidation level of formic acid, which is both a carboxylic acid and an aldehyde. When the elements of water are removed on attachment of formic acid to H_4PteGlu, only the CHO portion is attached. Similarly, the methylene group of CH_2-H_4PteGlu is at the oxidation level of formaldehyde (two molecules of water removed from formaldehyde hydrate), and the methyl group of 5-CH_3-H_4PteGlu is at the oxidation level of methanol.

B. PteGlu, H_2PteGlu, and H_4PteGlu

Although PteGlu, in which the pyrazine ring is aromatic, is active as a vitamin, it is not known to have a role as a coenzyme and is probably an artifact of the isolation procedure. The known pathway for the biosynthesis of folates leads to H_2PteGlu rather than PteGlu (Section IV). X-ray diffraction studies of crystals of PteGlu (Mastropaolo *et al.*, 1980) show that the C-4 and N-10 atoms are on the same side of the molecule, hydrogen-bonded to the same water molecule. Intermolecular stacking occurs between the pteridine and phenyl rings in the crystalline material and in solution (Pastore, 1971). Stacking interactions are not observed with 5-CHO-H_4PteGlu (Pastore, 1971) or H_4PteGlu (Poe and Hoogsteen, 1978) in solution.

H_2PteGlu is readily prepared from PteGlu by treatment with dithionite (Futterman, 1957; Blakley, 1960a; Friedkin *et al.*, 1962; Kawai and Scrimgeour, 1972). Proton NMR studies show conclusively that the structure of H_2PteGlu prepared in this manner is 7,8-H_2PteGlu (Pastore *et al.*, 1963). H_4PteGlu is usually prepared from PteGlu by catalytic hydrogenation (O'Dell *et al.*, 1947; Hoy *et al.*, 1951; Blakley, 1957; Kisliuk, 1957; Rabinowitz, 1960), which leads to a mixture of diastereoisomers at C-6 (Section II,F). Both H_2PteGlu and H_4PteGlu are labile to oxygen, the latter being more sensitive (Chippel and Scrimgeour, 1970). Depending on conditions, H_4PteGlu may cleave between the 6- and 10-positions or may be oxidized to PteGlu. 2-Mercaptoethanol (Mathews and Huennekens, 1960) or ascorbate (Bakerman, 1961) is commonly added to solutions of labile reduced folates to retard oxidation. 2-Mercaptoethanol is usually used in enzyme assays, and ascorbate is used in microbiological assays.

C. 5-CHO-H_4PteGlu, CH-H_4PteGlu, and 10-CHO-H_4PteGlu

There are three common derivatives of H_4PteGlu in which the single-carbon unit is at the oxidation level of formic acid. They are 10-CHO-H_4PteGlu, 5-CHO-H_4PteGlu, and CH-H_4PteGlu. The last-named derivative, CH-H_4PteGlu, can be prepared by dissolving H_4PteGlu in formic acid (Rowe, 1971) or by dissolving PteGlu in formic acid to form 10-CHO-PteGlu (Gordon *et al.*, 1948), followed by catalytic hydrogenation in formic or acetic acid (Rabinowitz, 1960).

CH-H_4PteGlu, which is oxygen labile, is converted to the stable 5-CHO-H_4PteGlu by heating under reducing conditions (Pohland *et al.*, 1951; Roth *et al.*, 1952; Temple *et al.*, 1979). CH-H_4PteGlu is converted to 10-CHO-H_4PteGlu by incubation at pH 8.0 (Rowe, 1971). This reaction has been studied in detail (Robinson and Jencks, 1967; Robinson, 1971). By bringing the pH to 3, one can convert both 10-CHO-H_4PteGlu and 5-CHO-H_4PteGlu to CH-H_4PteGlu (Rabinowitz, 1960).

5-CHO-H_4PteGlu, a stable derivative of H_4PteGlu, has been isolated from liver (Sauberlich and Baumann, 1948; Keresztesy and Silverman, 1951) and played a key role in the discovery that the enzymatically active form of PteGlu is H_4PteGlu. Although 5-CHO-H_4PteGlu is an effective precursor of H_4PteGlu in many biological systems (Goldin *et al.*, 1954; Blakley, 1969), a role for 5-CHO-H_4PteGlu in normal metabolism has not been demonstrated. Because it is formed from the known metabolite 10-CHO-H_4PteGlu on heating tissue extracts (Wittenberg *et al.*, 1962), the tissue levels reported in heat-treated extracts are not accurate. The best evidence that 5-CHO-H_4PteGlu may play a role in normal metabolism is the existence of the enzyme 5-CHO-H_4PteGlu cyclohydrolase, which catalyzes the ATP-dependent conversion of 5-CHO-H_4PteGlu to CH-H_4PteGlu (Section VII,A,12).

A simple chemical procedure for the synthesis of radioactive 5-CHO-H_4PteGlu of high specific activity has been described (Moran and Colman, 1982).

It has been shown by NMR studies that there are two interconvertible forms of 5-CHO-H_4-PteGlu in solution resulting from hindered rotation of the CHO group about the C—N bond (Pastore and Williamson, 1968). In the favored form the CHO carbonyl is oriented toward H-6 of the tetrahydropyrazine ring (Feeney *et al.*, 1980; Poe and Benkovic, 1980).

D. CH_2-H_4PteGlu

CH_2-H_4PteGlu is prepared by mixing formaldehyde and H_4PteGlu (Kisliuk, 1957; Blakley, 1960b; Kallen, 1971). It is relatively stable in alkaline solution but is labile under neutral or acid conditions (Osborn *et al.*, 1960). The mechanism of the reaction of formaldehyde with H_4PteGlu has been studied by Kallen and Jencks (1966b) and Benkovic (1980).

CH_2-H_4PteGlu is readily reduced by $NaBH_4$ to 5-CH_3-H_4PteGlu (Sakami and Ukstins, 1961; Keresztesy and Donaldson, 1961; Gupta and Huennekens, 1967; Blair and Saunders, 1970), which is more stable than H_4PteGlu. 5-CH_3-H_4PteGlu can be oxidized to 5-CH_3-5,6-H_2PteGlu, a compound readily reduced to the starting compound by treatment with 2-mercaptoethanol (Larrabee *et al.*, 1961; Donaldson and Keresztesy, 1962). 5-CH_3-H_4PteGlu is inactivated as a growth factor in tissue culture medium under conditions in which 5-CHO-H_4PteGlu is stable (Fujii, 1981). The mechanism of this inactivation is unknown.

10-CH_2-H_4PteGlu has been synthesized (Gupta and Huennekens, 1967), but it is not known to have a role in metabolism.

B. Folate Polyglutamates

Folates are found in tissues in the form of poly-γ-glutamyl derivatives of various glutamyl chain lengths (Section VIII,B). Folate polyglutamates are most often prepared by the procedure of Krumdieck and Baugh (1969), which employs a modification of the Merrifield solid-phase peptide synthetic method. This procedure leads to $PteGlu_n$, which can be converted to reduced metabolites by procedures analogous to those used for the corresponding $PteGlu_1$ derivatives (Mathews and Baugh, 1980; Kisliuk *et al.*, 1981). $PteGlu_n$ have also been prepared by solution chemistry (Godwin *et al.*, 1972; Goldman *et al.*, 1983).

C. Diastereoisomers of H_4PteGlu

H_4PteGlu contains an asymmetric center at C-6 of the tetrahydropyrazine ring and another at the α-carbon of the L-Glu residue (Fig. 1). The configuration at C-6 found in naturally occurring H_4PteGlu derivatives has the H at C-6 pointing up from the plane of the paper if H_4PteGlu is depicted as in Fig. 1 (Fontecilla-Camps *et al.*, 1979). In H_4PteGlu this is the *S*-configuration, as determined by the Cahn, Ingold, and Prelog conventions (Bentley, 1969). However, when the H on N-10 is substituted with C as in CH_2-H_4PteGlu, CH-H_4PteGlu, or 10-CHO-H_4PteGlu, the naturally occurring configuration at C-6 is designated *R*. In this chapter the terms *natural* and *unnatural* are used to designate the respective diastereoisomers.

The natural diastereoisomer at C-6 of H_4PteGlu and its corresponding poly-γ-glutamate forms can be prepared from the corresponding H_2PteGlu derivatives using NADPH and dihydrofolate reductase (Mathews and Huennekens, 1960; Kisliuk *et al.*, 1981). The unnatural diastereoisomer at C-6 can be prepared by catalytic reduction of $PteGlu_n$ to form the mixture of diastereoisomers of $H_4PteGlu_n$ followed by conversion of the natural forms to $H_2PteGlu_n$ using the thymidylate synthase reaction (Kisliuk *et al.*, 1974). $H_2PteGlu_n$ and the unnatural diastereoisomers CH_2-$H_4PteGlu_n$ are then separated by DEAE-cellulose chromatography.

The natural diastereoisomer of 10-CHO-$H_4PteGlu_3$ can be prepared from *Clostridium acidi-urici* and converted to $H_4PteGlu_3$ by arsenolysis catalyzed by 10-CHO-H_4PteGlu synthetase (Curthoys and Rabinowitz, 1972). This enzyme was also used to synthesize the natural diastereoisomer of CH-$H_4PteGlu_1$.

Diastereoisomers of 5-CHO-H_4PteGlu can be resolved by fractional crystallization (Feeney *et al.*, 1981; Temple *et al.*, 1981). Diastereoisomers of CH_2-H_4PteGlu are separated by DEAE-cellulose chromatography (Kaufman *et al.*, 1963; Horwitz *et al.*, 1969), whereas the corresponding diastereoisomers of H_4PteGlu are not. The probable reason for this difference in behavior is that the

N-5—N-10 methylene bridge holds the asymmetric center at C-6 and that at the carbon of L-Glu in a fixed relationship to one another as compared with the situation involving H_4PteGlu. Evidence for this view is provided by NMR studies. The tetrahydropyrazine ring of H_4PteGlu exists in solution as an equal mixture of two half-chair forms (Poe and Hoogsteen, 1978; Furrer *et al.*, 1978), whereas CH_2-H_4PteGlu has but one half-chair conformation (Poe *et al.*, 1979b). CH-H_4PteGlu also exists in a half-chair conformation (Khalifa *et al.*, 1979).

X-ray crystallographic studies show the differing relationships between the two asymmetric centers in the diastereoisomers of CH-H_4PteGlu (Fig. 2) (Fontecilla-Camps *et al.*, 1979). Whereas the pyrimidine, tetrahydropyrazine, imidazole, and benzene ring portions of the molecule are nearly mirror images, the conformation of the glutamate residues is different. In both diastereoisomers the benzene and heterocyclic rings are almost coplanar, with all component atoms lying within 0.35 Å of a common plane. Bond lengths indicate that there is a

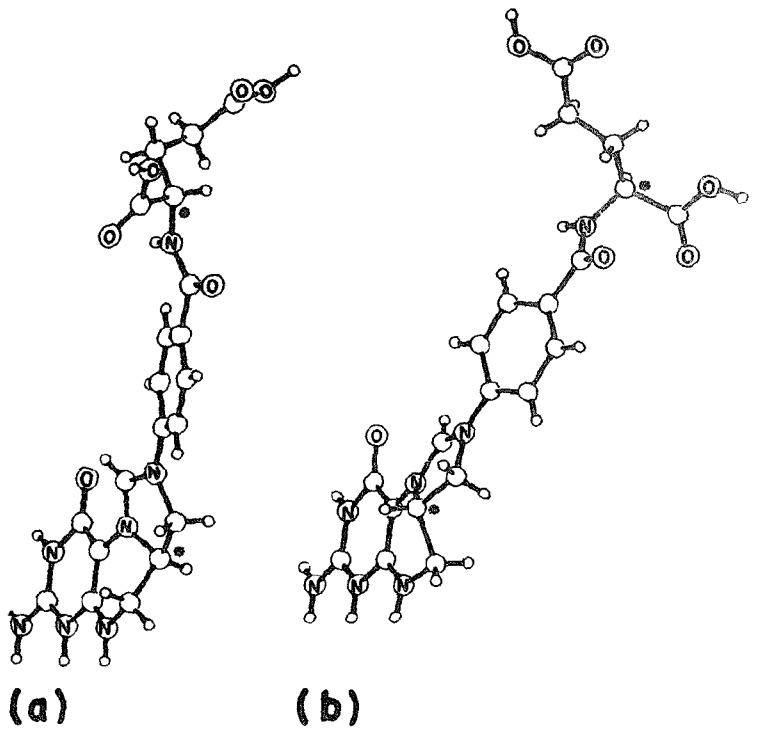

FIG. 2. Crystal structures of the (a) natural and (b) unnatural diastereoisomers of methenyl tetrahydrofolate. From Fontecilla-Camps *et al.*, 1979. Copyright 1979 American Chemical Society.

resonating conjugated system through the pyrimidine ring, the imidazole ring, and the benzene ring.

Instances of enzyme inhibition by the unnatural diastereoisomer, which is usually present in assay mixtures, are listed in Table II. In the case of glycinamide ribonucleotide formyltransferase, the inhibitory potency of the unnatural diastereoisomer of 10-CHO-H_4PteGlu led to an erroneous assessment of substrate specificity (see Section VII,A,10). With *Lactobacillus casei* thymidylate synthase, inhibition by the unnatural diastereoisomer of CH_2-H_4PteGlu is enhanced as the length of the polyglutamate chain increases (Kisliuk *et al.*, 1974).

A mixture of diastereoisomers of 5,10-methylenetetrahydroaminopterin shows synergistic inhibition of the growth of *Pediococcus cerevisiae*, implying that each form has a different site of action (Horwitz *et al.*, 1969).

The configuration of C-6 of H_4-biopterin (Matsuura *et al.*, 1980) and 6-methyl-5,6,7,8-tetrahydropterin (Armarego *et al.*, 1980), both prepared from the corresponding dihydro forms by incubation with dihydrofolate reductase and NADPH, is the same as that of the natural configuration of H_4PteGlu.

G. Dissociation Constants

Acid dissociation constants for folate derivatives are given in Table III (Kallen and Jencks, 1966a; Poe, 1977). This information is useful in interpreting the mechanism of the interaction of single-carbon units with folates as well as the interaction of folates with enzymes. For example, CH_2-H_4PteGlu can give rise to an electrophilic iminium ion ($^-CH_2{=}N^+$), which is located on N-5 because of the relatively high pK of N-5 relative to N-10. This iminium ion is postulated to play a role in reactions involving the transfer of this group such as those catalyzed by serine hydroxymethyltransferase and thymidylate synthase (Benkovic and Bullard, 1973; Benkovic, 1980) and CH_2-H_4PteGlu reductase (Matthews, 1982). The high pK of the N1 of methotrexate relative to the N-1 of folates is known to play an important role in the tight binding of methotrexate to dihydrofolate reductase (Erickson and Mathews, 1972; Sapirstein *et al.*, 1978; Cocco *et al.*, 1981).

TABLE II

ENZYME INHIBITION BY THE UNNATURAL DIASTEREOISOMERS OF TETRAHYDROFOLATE COENZYMES

Inhibitor	Enzyme	Source	Reference
10-CH_3-H_4PteGlu	Thymidylate synthase	*Lactobacillus casei*	Leary *et al.* (1974)
CH_2-H_4PteGlu$_n$	Thymidylate synthase	*L. casei*	Kisliuk *et al.* (1974)
CH_2-H_4PteGlu	Methylenetetrahydrofolate dehydrogenase	*Escherichia coli*	Scott and Donaldson (1964)
10-CHO-H_4PteGlu	Glycinamide ribonucleotide formyltransferase	Chicken liver	Smith *et al.* (1981a)
H_4PteGlu	Formiminotetrahydrofolate cyclodeaminase	Pig liver	MacKenzie and Baugh (1983)
H_4PteGlu	10-Formyltetrahydrofolate dehydrogenase	Pig liver	Kutzbach and Stokstad (1971b)

TABLE III

DISSOCIATION CONSTANTS OF FOLATE DERIVATIVES[a]

Compound	pK'_a Amide (N-3—C-4)	N-1	N-5	N-10	α-COOH	γ-COOH
PteGlu	8.4	2.4	<1.5	0.2	—	—
H_2PteGlu	9.5	1.4	3.8	0.28	—	—
H_4PteGlu	10.5	1.2	4.8	1.3	3.5	4.8
Methotrexate	—	5.7	<1.5	0.5	3.4	4.7

[a]From Kallen and Jencks (1966a) and Poe (1977).

H. Ultraviolet Absorbance Spectra

The UV absorbance spectra are useful for identification and for enzyme assays. For example, the difference in absorbance at 340 nm between H_2PteGlu and H_4PteGlu is widely used in assays for dihydrofolate reductase (Mathews *et al.*, 1963) and thymidylate synthase (Wahba and Friedkin, 1962). In the dihydrofolate reductase reaction, the concurrent changes in absorbance at 340 nm brought about by the conversion of NADPH to NADP are additive with those associated with the conversion of H_2PteGlu to H_4PteGlu.

The UV absorbance spectra of most folate derivatives can be found in the review by Rabinowitz (1960). The spectra of 5-CH_3-H_4PteGlu and 5-CH_3-5,6-H_2PteGlu, which were discovered subsequently, are given by Donaldson and Keresztesy (1962) and Larrabee *et al.* (1961). The molar absorption coefficients of some PteGlu derivatives are listed in Table IV. A more extensive list is given by Blakley (1969).

III. Folate Determination

A. Introduction

The determination of folates in biological materials is difficult because (*a*) their concentrations are low (5–15 μg/g liver; Bird *et al.*, 1965), (*b*) there are at least six different single-carbon unit derivatives potentially present (Table I), (*c*) there are three oxidation states of the pyrazine ring (Table I), (*d*) there are various

TABLE IV

MOLAR ABSORPTION COEFFICIENTS OF FOLATE DERIVATIVES

Compound	pH	Wavelength (nm)	Molar absorption coefficient	References
PteGlu	7.0	282	27,600	Rabinowitz (1960)
H_2PteGlu	7.0	282	28,400	Blakley (1960a)
H_4PteGlu	7.2	298	28,400	Blakley (1960b)
5-CHO-H_4PteGlu	7.0	285	33,000	Uyeda and Rabinowitz (1965)
5-CHO-H_4PteGlu	7.0	258	19,000	Kay *et al.* (1960)
5,10-CH^+-H_4PteGlu	2.0	352	25,000	Rabinowitz (1960)
5,10-CH_2-H_4PteGlu	7.2	294	32,000	Blakley (1960b)
5-CH_3-H_4PteGlu	7.0	290	31,700	Gupta and Huennekens (1967)
Homofolate	13.0	281	19,400	Kisliuk (1971)
H_4-Homofolate	7.2	282	19,700	Kisliuk (1971)
H_2-Homofolate	7.2	295	20,500	Kisliuk (1971)
Aminopterin	13.0	284	26,400	Seeger *et al.* (1949)
H_4-Aminopterin	7.0	289	27,000	Kisliuk and Levine (1964)
H_2-Aminopterin	7.0	296	28,300	Kisliuk and Levine (1964)
Methotrexate	13.0	302	22,000	Seeger *et al.* (1949)

polyglutamate chain lengths (see Table XV), (*e*) various reduced folates differ in their stability to oxygen, and (*f*) hydrolases are present in many tissues that catalyze the cleavage of γ-glutamyl bonds. An ideal goal for tissue folate analyses would be to measure the various single-carbon unit derivatives, the oxidation level of the pyrazine ring as well as the polyglutamate chain length associated with each folate derivative. The extraction procedure should therefore (*a*) liberate bound forms quantitatively, (*b*) avoid extremes of pH and heat, (*c*) be carried out in the presence of reducing agents, (*d*) prevent enzymatic degradation, and (*e*) avoid photodecomposition (Baugh and Krumdieck, 1971).

In order to verify the accuracy of the determination one would have to determine the recovery of standard samples of each potential folate derivative from the tissue in question. It is presently impractical to carry out this type of recovery experiment. Tissues are usually extracted with hot 1% sodium ascorbate solutions (Brody *et al.*, 1982; Yin *et al.*, 1983) but this may introduce errors (Wilson and Horne, 1983). Anaerobic acid treatment has also been used (Eto and Krumdieck, 1981). Treatment with γ-glutamyl hydrolase (conjugase) converts polyglutamate forms to monoglutamate forms, which can be separated and determined by the methods listed in Table V.

B. MICROBIOLOGICAL ASSAY

The three bacterial species ordinarily used to assay for folates are *Pediococcus cerevisiae*, *Streptococcus faecium*, and *Lactobacillus casei* (Flynn *et al.*, 1951; Bakerman, 1961; Baker *et al.*, 1971; Cooperman, 1971). Each organism differs with respect to the types of folate derivatives that will support growth (Table VI). *Pediococcus cerevisiae* is remarkable in that it requires an H_4PteGlu derivative and does not grow on PteGlu or H_2PteGlu even though it contains dihydrofolate reductase, which can catalyze the reduction of PteGlu and H_2PteGlu to H_4PteGlu (Mandelbaum-Shavit and Grossowicz, 1970, 1975; Mandelbaum-Shavit, 1976). *Pediococcus cerevisiae* cannot transport PteGlu or H_2PteGlu through its cell membrane. Certain mutants of this organism transport PteGlu (Mandelbaum-Shavit, 1976). *Pediococcus cerevisiae* also does not grow on 5-CH_3-H_4PteGlu, but this is not due to a transport deficiency (Mandelbaum-Shavit and Grossowicz, 1975). Apparently, the organism cannot remove the 5-CH_3 group. H_4PteGlu$_2$ and 5-CHO-H_4PteGlu$_2$ support growth, but activity falls off sharply with the corresponding triglutamate derivatives (Table VI). The standard compound usually used for assays with *P. cerevisiae* is 5-CHO-H_4PteGlu$_1$.

TABLE V

METHODS OF FOLATE ANALYSIS

Objective	Method	References
Separation	DEAE-Cellulose chromatography	Wittenberg *et al.* (1962), Rao and Noronha (1978), Brody *et al.* (1982)
	DEAE-Sephadex chromatography	Nixon and Bertino (1971)
	Thin-layer chromatography	Scott (1980)
	Gel filtration	Shin *et al.* (1972), Kas and Cerna (1980)
	High-performance liquid chromatography	Archer and Reed (1980), Cashmore *et al.* (1980), Horne *et al.* (1981), McMartin *et al.* (1981)
Determination	Microbiological	Table VI
	Ligand binding	Waxman and Schreiber (1980)
	Immunological	Ricker and Stollar (1967), Raso and Schreiber (1975)
	Chemical	Eto and Krumdieck (1981)
	Enzymatic	Jaenicke (1971)
Polyglutamate chain length determination	Electrophoretic	Priest *et al.* (1980a, 1981a), Priest and Mangum (1981)
	Chemical	Foo *et al.* (1980), Eto and Krumdieck (1982)
	DEAE-Cellulose chromatography	Krumdieck and Baugh (1969), Brody *et al.* (1982)
	High-performance liquid chromatography	Jolivet and Schilsky (1981), Shane (1982)

TABLE VI

UTILIZATION OF FOLATES AS GROWTH FACTORS FOR MICROORGANISMS[a]

Compound	*Pediococcus cerevisiae* (ATCC 8081)	*Streptococcus faecium* (ATCC 8043)	*Lactobacillus casei* (ATCC 7469)
Pte	−	+	−
$PteGlu_1$	−	+	+
$PteGlu_2$	−	+	+
$PteGlu_3$	−	−	+
$PteGlu_4$	−	−	±[c]
$PteGlu_5$	−	−	±[c]
$PteGlu_6$	−	−	±[c]
$PteGlu_7$	−	−	±[c]
$H_2PteGlu_1$	−	+	+
$H_4PteGlu_1$	+	+	+
$H_4PteGlu_2$	+	+	+
$H_4PteGlu_3$[b]	−	−	+
$H_4PteGlu_5$[b]	−	−	±
5-CHO-$H_4PteGlu_1$	+	+	+
5-CHO-$H_4PteGlu_2$	+	+	+
5-CHO-$H_4PteGlu_3$	−	−	+
5-CH_3-$H_4PteGlu_1$	−	−	+

[a] Data from Johns and Bertino (1965), Baugh and Krumdieck (1971), Shiota (1971), Cooperman (1971), Kas and Cerna (1980), and Kisliuk (1981).

[b] A. Gaumont, R. L. Kisliuk, and C. M. Baugh, unpublished work.

[c] $PteGlu_4$, $PteGlu_5$, $PteGlu_6$, and $PteGlu_7$ are 66, 20, 4, and 3% as active, respectively, as $PteGlu_1$ for *L. casei*. Shane and Stokstad (1976).

Streptococcus faecium grows on most $PteGlu_1$ forms, except 5-CH_3-H_4PteGlu (Table VI). As with *P. cerevisiae*, it can accumulate 5-CH_3-H_4PteGlu from the medium (Mandelbaum-Shavit and Kisliuk, 1979). It also does not grow on polyglutamate forms with more than two glutamate residues.

Lactobacillus casei differs from the other two organisms in that (*a*) it can grow on 5-CH_3-H_4PteGlu because it can remove the 5-CH_3 group oxidatively (Shane and Stokstad, 1977a,b) and (*b*) it gives growth comparable to that with $PteGlu_1$ when offered polyglutamate forms with three glutamate residues. Diminishing activity is seen as the glutamate chain becomes longer (Table VI).

Lactobacillus casei is used to measure serum folate, which is predominantly 5-CH_3-$H_4PteGlu_1$ (Cooperman, 1971). A ligand-binding assay using radioactive folate and a folate-binding protein is also used for this purpose (Waxman and Schreiber, 1980). *Lactobacillus casei* determinations tend to be 15% higher than those given by the ligand-binding assay. Values of 0 to 3 ng/ml of serum indicate folate deficiency, whereas values greater than 8 ng/ml are normal. Intermediate values are indeterminate.

C. Determination of Polyglutamate Chain Length

Four methods used to determine polyglutamate chain length are (*a*) amino acid analysis of pure samples (Pfiffner *et al.*, 1946; Curthoys and Rabinowitz, 1972; Rao and Noronha, 1978), (*b*) chemical degradation of $PteGlu_n$ derivatives to *p*-aminobenzoyl-Glu_n followed by chromatographic separation and determination of the latter compounds (Foo *et al.*, 1980), (*c*) DEAE-cellulose chromatography of $PteGlu_n$ (Table V), and (*d*) electrophoretic separation of ternary complexes of CH_2-$H_4PteGlu_n$, [^{3}H]FdUMP, and *L. casei* thymidylate synthase (Priest *et al.*, 1980a).

The chemical degradation of $PteGlu_n$ derivatives to *p*-aminobenzoyl-Glu_n by treatment with $KMnO_4$ or Zn-HCl would be expected to eliminate ambiguities caused by different one-carbon constituents and different oxidation states of the pyrazine ring and result in a measure of polyglutamate chain length in the total folate pool. Although either $KMnO_4$ or Zn-HCl treatment appeared to suffice, it turned out that 5-CH_3-$H_4PteGlu_n$, often the major tissue folates, are not completely cleaved by either procedure (Maruyama *et al.*, 1978; Lewis and Rowe, 1979; Baugh *et al.*, 1979), so that more effective methods had to be devised (Foo *et al.*, 1980; Eto and Krumdieck, 1981). These methods include (*a*) acid treatment, which converts 10-CHO-$H_4PteGlu_n$, 5-CHO-$H_4PteGlu_n$, and 5-CHNH-$H_4PteGlu_n$ to CH-$H_4PteGlu_n$; (*b*) reduction with $NaBH_4$, which converts CH-$H_4PteGlu_n$ to 5-CH_3-$H_4PteGlu_n$, joining the 5-CH_3-$H_4PteGlu_n$ already present; and (*c*) oxidation of 5-CH_3-$H_4PteGlu_n$ to CH_3-5,6-$H_2PteGlu_n$, which are cleaved in acid to *p*-aminobenzoyl-Glu_n. CH-$H_4PteGlu_n$, $H_2PteGlu_n$, and $H_4PteGlu_n$ are also cleaved to *p*-aminobenzoyl-Glu_n by these procedures. Thus, all of the common tissue folates are converted to *p*-aminobenzoyl-Glu_n, which can be resolved according to polyglutamate chain length up to *p*-aminobenzoyl-Glu_{11} (Shane, 1982).

D. Examples of Tissue Folate Determinations

Eto and Krumdieck (1981) used sequential acidification, oxidation, and reduction to identify three folate pools in rat liver: (*a*) CH_2-$H_4PteGlu_n$ and $H_4PteGlu_n$; (*b*) CH_3-$H_4PteGlu_n$; and (*c*) CH-$H_4PteGlu_n$, 10-CHO-$H_4PteGlu_n$, 5-CHO-$H_4PteGlu_n$, and CHNH-$H_4PteGlu_n$. The diazotized derivatives of *p*-aminobenzoyl-Glu_n derived from each pool were determined using reversed-phase HPLC. As little as 20 pmol of the diazotized derivatives could be detected. Partial hepatectomy alters the proportions of folyl polyglutamates (Eto and Krumdieck, 1982); Glu_6 and Glu_7 increase at the expense of Glu_4 and Glu_5. CH_3-$H_4PteGlu_6$ accounts for most of the increase in Glu_6, and formyl-level compounds account for most of the increase in Glu_7. These changes are probably related to the regulation of one-carbon metabolism.

Brody *et al.* (1982) combined the use of DEAE-cellulose chromatography, hog kidney γ-glutamyl hydrolase, and microbiological assay to study folate

metabolism in rat liver. Within a series of derivatives with the same folate moiety, the longer the polyglutamate chain, the greater the affinity for DEAE-cellulose. However, elution from DEAE-cellulose is altered by the state of oxidation of the pyrazine ring (Kisliuk *et al.*, 1974) as well as by the various one-carbon substituents (Brody *et al.*, 1982). The rats used in this study were maintained on a diet low in methionine and were also treated with N_2O gas, which would be expected to raise the levels of 5-CH_3-H_4PteGlu$_n$ (Section VI,C). At the Glu_5 level, 5-CH_3-H_4PteGlu$_5$ was the major folate, there being only small amounts of H_4PteGlu$_5$. At the Glu_6 level, the two forms were about equal and, at the Glu_7 level, H_4PteGlu predominated. It appears that metabolic function differs with polyglutamate chain length. After methionine administration the levels of 5-CH_3-H_4PteGlu$_{5-7}$ decreased sharply, as would be anticipated (Section VI,C).

Using techniques similar to those of Brody *et al.* (1982), Yin *et al.*, (1983) showed that mouse sarcoma 180 cells have larger folate pools with longer glutamate chains than do human carcinoma cells (Hep-2). These results provide a reasonable explanation for the greater susceptibility of the mouse cells to inhibition by 5-fluorouracil, which after conversion to 5-fluorodeoxyuridylate binds to thymidylate synthase more firmly in the presence of CH_2-H_4PteGlu$_n$ (Section VII,A,4,f).

Electrophoresis of the ternary complex of CH_2-H_4PteGlu$_n$, fluorodeoxyuridylate, and *L. casei* thymidylate synthase (Priest *et al.*, 1980a, 1981a) has proved useful in determining (*a*) the length of the polyglutamate chains of CH_2-H_4PteGlu in tissues (Priest *et al.*, 1981a, 1983), (*b*) the relative affinity of CH_2-H_4PteGlu$_n$ for thymidylate synthase (Priest and Mangum, 1981), (*c*) the activity of folyl-γ-glutamyl hydrolases (Priest *et al.*, 1982), and (*d*) the activity of folylpolyglutamate synthetase (Priest *et al.*, 1981b).

IV. Biosynthesis of H_2PteGlu

A. Enzymic Reactions

Guanosine triphosphate is the common precursor of H_2PteGlu, riboflavin, and H_2-biopterin (Shiota, 1971; Brown, 1982). The reactions leading from GTP to H_2PteGlu are outlined in Fig. 3, and the names of the enzymes catalyzing these reactions are listed in Table VII. Reaction 1, catalyzed by GTP cyclohydrolase I, results in a striking rearrangement of GTP to form H_2-neopterin triphosphate. The GTP cyclohydrolase I from *E. coli* has a molecular weight of 210,000, is heat stable, and consists of four identical subunits (Yim and Brown, 1976). GTP cyclohydrolase I has also been found in *Lactobacillus plantarum* (Jackson and Shiota, 1971), *Drosophila* (Dorsett *et al.*, 1979), chicken kidney (Tanaka *et al.*, 1981), hamster kidney (Eto *et al.*, 1976), and rat brain (Lee *et al.*, 1979). GTP cyclohydrolase II catalyzes a similar reaction on the pathway to riboflavin

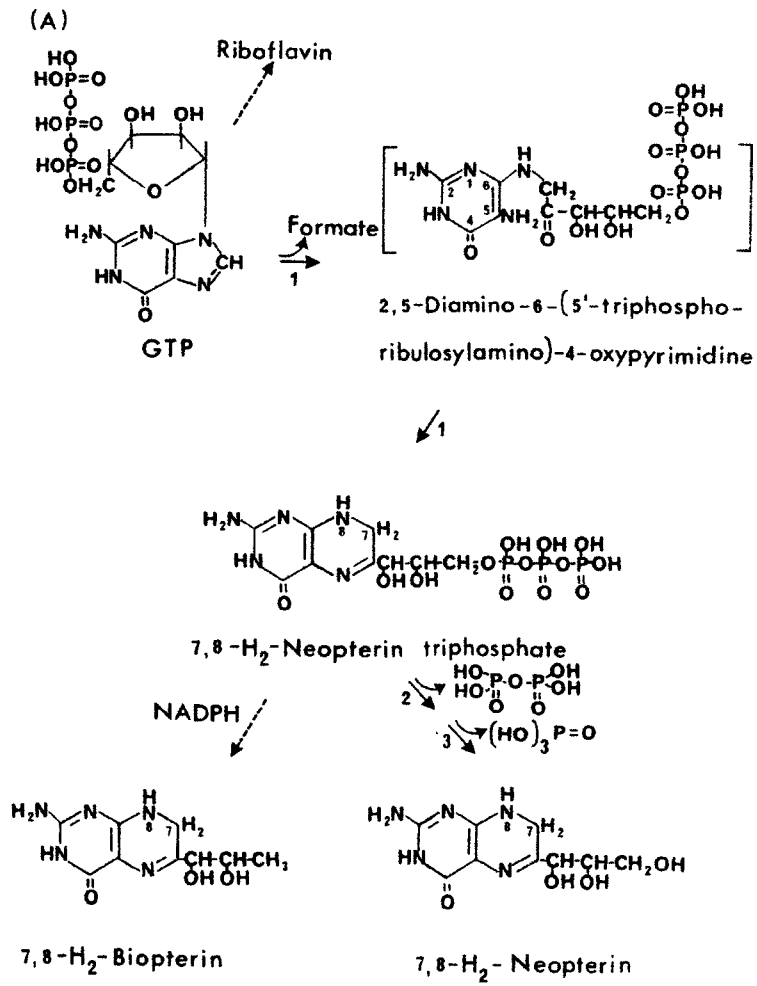

FIG. 3. Reactions involved in the biosynthesis of dihydrofolate. (A) Biosynthesis of dihydroneopterin. (B) Conversion of dihydroneopterin to dihydrofolate (see p. 16). The enzymes catalyzing the numbered reactions are listed in Table VII. (*p*-AB, *p*-aminobenzoic acid).

(Brown, 1982), but the product in this instance is a phosphoribosylpyrimidine, which is then reduced to a phosphoribitylpyrimidine.

H_2-Neopterin triphosphate is at the branch point of pathways leading to H_2PteGlu or to other pterins including H_2-biopterin (Fukushima and Shiota, 1974; Eto *et al.*, 1976), sepiapterin (Tanaka *et al.*, 1981), drosopterin (Wiederrecht *et al.*, 1981), and butterfly wing pigments (Watt, 1967). On the H_2PteGlu

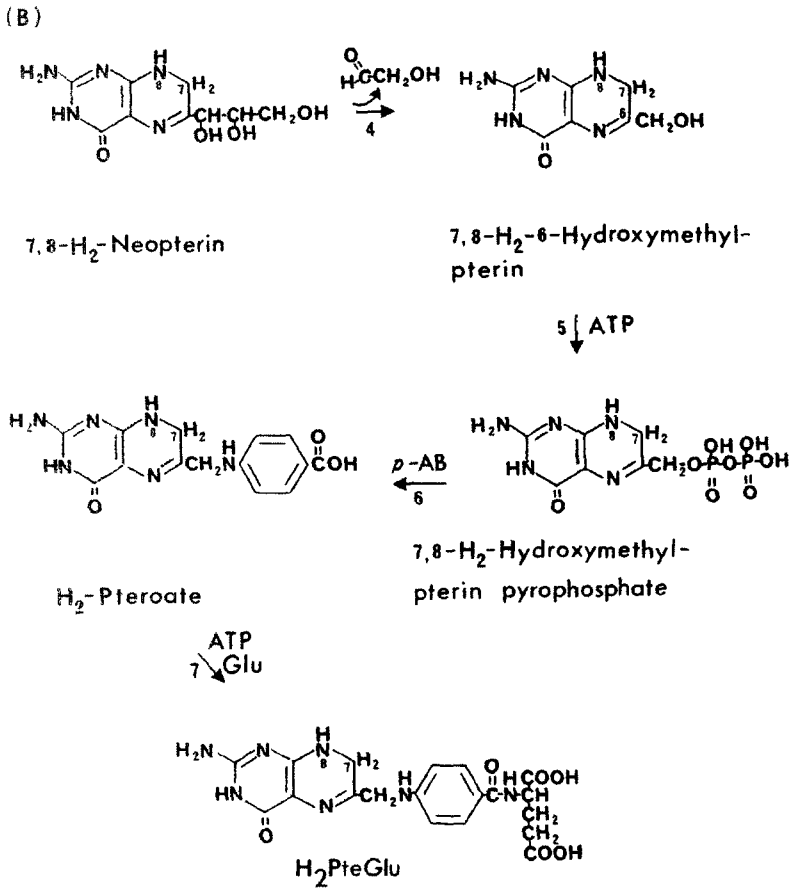

FIG. 3. (*continued*).

pathway, H_2-neopterin triphosphate loses a pyrophosphate group in a reaction catalyzed by H_2-neopterin triphosphate pyrophosphohydrolase. In *E. coli* this enzyme is specific for H_2-neopterin triphosphate and does not hydrolyze the common nucleoside triphosphates (Brown, 1982). The removal of the third phosphate is catalyzed by a nonspecific phosphatase. Glycolaldehyde is then cleaved from the side chain of H_2-neopterin, yielding H_2-hydroxmethylpterin. The hydroxymethyl group is pyrophosphorylated by ATP, yielding H_2-hydroxymethylpterin pyrophosphate, which in turn reacts with *p*-aminobenzoate to give H_2Pte. Sulfonamides prevent the incorporation of *p*-aminobenzoate into H_2Pte

by virtue of their substrate activity with dihydropteroate synthase (Brown, 1962; Shiota *et al.*, 1964). The H_2Pte-sulfonamide derivatives formed are inert as folate percursors. H_2Pte finally reacts with ATP and glutamic acid to yield H_2PteGlu (Fig. 3).

The H_2PteGlu synthetases from *Corynebacterium* and *E. coli* are bifunctional (Shane, 1980; Ferone and Warskow, 1983). In addition to catalyzing the formation of H_2PteGlu from H_2Pte, glutamic acid, and ATP, these enzymes possess folylpolyglutamate synthetase activity. Rat liver folylpolyglutamate synthetase does not catalyze the synthesis of H_2PteGlu from H_2Pte (McGuire *et al.*, 1980).

B. Metabolic and Chemotherapeutic Aspects

Animals can carry out the synthesis of H_2-biopterin, the cofactor for phenylalanine hydroxylase (Kaufman, 1979), but not the synthesis of H_2PteGlu (Fukushima and Shiota, 1974; Eto *et al.*, 1976; Tanaka *et al.*, 1981; Yoshioka *et al.*, 1983). Defective biopterin synthesis leading to hyperphenylalaninemia has been reported in humans (Niederwiesser *et al.*, 1982). In one case GTP cyclohydrolase is missing (Niederwieser *et al.*, 1983). Apparently, animals do not contain H_2-neopterin triphosphate pyrophosphohydrolase or any of the subsequent enzymes on the pathway to H_2PteGlu. The pterins excreted in the urine of patients with cancer (Halpern *et al.*, 1977; Bichler *et al.*, 1982; Rao *et al.*, 1983) may arise from the biopterin pathway (Fig. 3).

Because many pathogens synthesize H_2PteGlu and do not have a transport system for folates, enzymes 3, 4, 5, 6, and 7 (Table VII) are potential targets for selective chemotherapeutic agents, which could enhance the activity of trimethoprim and sulfonamides. Some analog development along these lines has been reported (Kisliuk *et al.*, 1967; Mathis and Brown, 1970; Wood, 1975; Ferone and Webb, 1975; Ho *et al.*, 1976; Zimmerman *et al.*, 1977; Ponngsamart *et al.*, 1981).

TABLE VII

ENZYMES INVOLVED IN THE SYNTHESIS OF DIHYDROFOLATE[a]

1. GTP cyclohydrolase I
2. Dihydroneopterin triphosphate pyrophosphohydrolase
3. Dihydroneopterin phosphate phosphatase
4. Dihydroneopterin aldolase
5. Dihydrohydroxymethylpterin pyrophosphokinase
6. Dihydropteroate synthase
7. Dihydrofolate synthetase

[a]See Fig. 3.

V. Biodegradation

After intravenous administration of [2-^{14}C]folate to a human volunteer, absorption into tissues was greater than 90% (Krumdieck *et al.*, 1978). Urinary excretion showed a bimodal pattern: one half-life of 32 h and a second of 100 days. Biopterin, *erythro*-neopterin, and *threo*-neopterin isolated from the urine were not radioactive, whereas pterin and isoxanthopterin were radioactive, showing that some of the radioactive folate had been cleaved. Fecal excretion proved to be a significant route of folate elimination. In rats the *p*-aminobenzoyl-Glu portion of folate is excreted in the acetylated form (Murphy *et al.*, 1976). The microflora may play a role in folate degradation in rats, because administration of antibiotics reduced catabolite formation (Pheasant *et al.*, 1981).

A likely pathway for the degradation of folate involves reduction to H_4PteGlu, which cleaves spontaneously to a pterin and *p*-aminobenzoyl-Glu (Futterman and Silverman, 1957; Saleh *et al.*, 1981, 1982; Pheasant *et al.*, 1983).

An interesting isotope effect was observed in metabolic studies of [^{3}H]- and [^{14}C]folates (Connor *et al.*, 1980). Excreted folates contained more ^{3}H than ^{14}C relative to the compound administered due to enhanced intestinal absorption of the ^{3}H-labeled compound. Isotope effects were also observed on ion-exchange chromatography.

The enzymes from microorganisms that catalyze the degradation of folate and methotrexate have been reviewed (Kalghatgi and Bertino, 1981). One such enzyme, carboxypeptidase G, catalyzes the hydrolysis of the amide bond and can be used to induce folate deficiency or to inactivate methotrexate *in vivo*.

VI. Metabolism

A. Introduction

An outline of the metabolic interrelationships between folate derivatives is given in Fig. 4. The names of the 17 enzymes catalyzing these interconversions are listed in Table VIII. Additional reactions known to require folate coenzymes are listed in Table IX.

B. Source and Fate of Single-Carbon Units

One-carbon units are derived from serine, glycine (Ogur *et al.*, 1977; Dev and Harvey, 1982), formiminoglutamate (Silverman and Pitney, 1958), or formate (Case and Benevenga, 1977) and are used to form the methyl groups of methionine and thymidylate, are used to form C-2 and C-8 of inosinic acid, or are oxidized to CO_2. Serine, the major source of single-carbon units, arises from phosphoglycerate, formiminoglutamate from histidine, and formate from the oxidation of methyl groups (Blakley, 1969). Formate also arises from C-1 of the ribose moiety of methylthioadenosine, which arises as a by-product of the conversion of adenosylmethionine to polyamines (Trackman and Abeles, 1981). 10-CHO-H_4PteGlu, via its interaction with the enzyme 10-CHO-H_4PteGlu dehydrogenase, provides a mechanism by which excess single-carbon units can be removed as CO_2 (Scrutton and Beis, 1979). Folate administration to animals enhances the conversion of ingested methanol and formate to CO_2, diminishing methanol toxicity (Noker *et al.*, 1980).

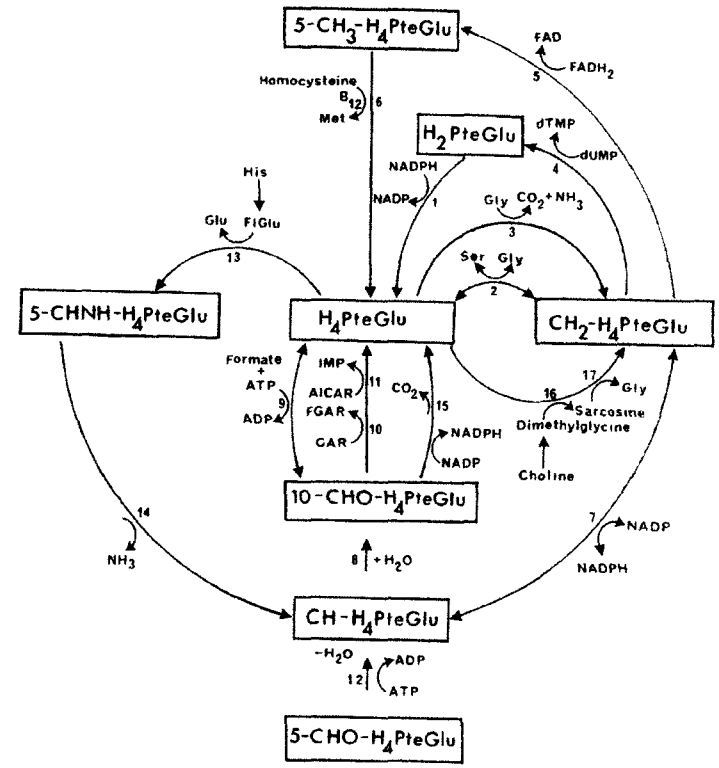

FIG. 4. Metabolic interrelationships of tetrahydrofolate coenzymes. The enzymes catalyzing the numbered reactions are listed in Table VIII. Abbreviations: GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; AICAR, aminoimidazolecarboxamide ribonucleotide; FIGlu, formiminoglutamic acid; IMP, inosine monophosphate (inosinic acid).

C. Regulatory Role of Methionine, 5-CH_3-H_4PteGlu, and Vitamin B_{12}

Methionine is a key metabolite regulating the metabolism of single-carbon units (Krebs *et al.*, 1976; Jägerstad *et al.*, 1980; Brody *et al.*, 1982; Eells *et al.*, 1982; Stokstad *et al.*, 1983). In the form of adenosylmethionine, it regulates the

TABLE VIII

FOLATE ENZYMES OF WIDESPREAD DISTRIBUTION[a]

1. Dihydrofolate dehydrogenase (dihydrofolate reductase) (EC 1.5.1.3)
2. Serine hydroxymethyltransferase (EC 2.1.2.1)
3. Glycine synthase (glycine cleavage system) (EC 2.1.2.10)
4. Thymidylate synthase (EC 2.1.1.45)
5. Methylenetetrahydrofolate reductase (EC 1.1.99.15)
6. Tetrahydropteroylglutamate methyltransferase (5-methyltetrahydrofolate-homocysteine methyltransferase) (EC 2.1.1.13)
7. Methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5)
8. Methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9)
9. 10-Formyltetrahydrofolate synthetase (EC 6.3.4.3)
10. Phosphoribosylglycinamide formyltransferase (glycinamide ribonucleotide formyltransferase) (EC 2.1.2.2)
11. Phosphoribosylaminoimidazolecarboxamide formyltransferase (aminoimidazolecarboxamide ribonucleotide formyltransferase) (EC 2.1.2.3)
12. Methenyltetrahydrofolate synthetase (5-formyltetrahydrofolate cyclodehydrase) (EC 6.3.3.2)
13. Glutamate formiminotransferase (formiminoglutamate:tetrahydrofolate formiminotransferase) (EC 2.1.2.5)
14. Formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4)
15. 10-Formyltetrahydrofolate dehydrogenase (EC 1.5.1.6)
16. Phosphoglycerate dehydrogenase (EC 1.5.99.2)
17. Sarcosine dehydrogenase (EC 1.5.99.1)

[a] The metabolic interrelationships of these enzymes are shown in Fig. 4. The names listed are those recommended by the International Union of Biochemistry (Enzyme Nomenclature Committee, 1979). When an alternative name is used in the text, it is given in parentheses.

levels of 5-CH_3-H_4PteGlu and H_4PteGlu, which in turn regulate the distribution of single-carbon units. As methionine concentration increases, 5-CH_3-H_4PteGlu levels decrease because of decreased synthesis as well as increased demethylation. Decreased synthesis of 5-CH_3-H_4PteGlu results in increased availability of its precursor, CH_2-H_4PteGlu, and the increased demethylation yields H_4PteGlu. The CH_2-H_4PteGlu thus formed can be used for the synthesis of thymidylate, serine, or inosinic acid, and the H_4PteGlu can be used as a formimino group acceptor. As methionine concentration decreases, synthesis of 5-CH_3-H_4PteGlu is enhanced and its demethylation is decreased, which results in a shortage of CH_2-H_4PteGlu and H_4PteGlu. If the methionine level falls so low that the remethylation of homocysteine from 5-CH_3-H_4PteGlu cannot be maintained adenosylmethionine is an absolute requirement for the 5-CH_3-H_4PteGlu-homocysteine methyltransferase reaction (Mangum and Scrimgeour, 1962; Kisliuk, 1964; Taylor and Weissbach, 1973)]. 5-CH_3-H_4PteGlu accumulates and a deficiency of the other folate coenzymes ensues. Such a situation obtains in perfused rat liver or in isolated rat hepatocytes due to methionine leakage from the cells and can be detected because added histidine cannot be metabolized beyond formiminoglutamate, which accumulates due to inadequate amounts of free

TABLE IX

FOLATE ENZYMES OF SPECIALIZED FUNCTION

Enzyme	Source	Function	References
Trimethylsulfonium methyltransferase	*Pseudomonas*	Use of trimethylsulfonium as a carbon source	Wagner *et al.* (1967)
5-CH_3-H_4Pteglu-pyruvate methyltransferase	*Clostridium thermoaceticum*	Acetate formation	Drake *et al.* (1981)
Ribothymidyl synthase	*Streptococcus faecalis*	Methylation of uracil in tRNA	Delk *et al.* (1980)
Ketopantoate hydroxymethyltransferase	*Escherichia coli*	Synthesis of an intermediate on the pathway to pantothenate	Powers and Snell (1976)
Deoxyuridylate hydroxymethyltransferase	*Bacillus subtilis* phage	Hydroxymethyluracil is a component of the phage DNA	Hemphill and Whiteley (1975), Kunitani and Santi (1980)
Deoxycytidylate hydroxymethyltransferase	*E. coli* phage	Hydroxymethylcytosine is a component of the phage DNA	Mathews *et al.* (1964)
Formiminoglycine formiminotransferase	*Clostridum acidi-urici*	Use of purines as a carbon and nitrogen source	Rabinowitz (1960)
Methionyl-tRNA formyltransferase	*E. coli* and animal mitochondria	Initiation of protein synthesis by formylmethionyl-tRNA	Dickerman and Smith (1970), Halbreich and Rabinowitz (1971)
Enzyme-bound valine formyltransferase	*Bacillus brevis*	Synthesis of the peptide antibiotic gramicidin A	Akashi and Kurahashi (1977, 1978)

H_4PteGlu (Krebs *et al.*, 1976). Formiminoglutamate excretion is abolished by the addition of methionine.

Those conditions that diminish free H_4PteGlu levels, such as (*a*) methionine deficiency, (*b*) vitamin B_{12} deficiency [vitamin B_{12} is a coenzyme for mammalian 5-CH_3-H_4PteGlu-homocysteine methyltransferase (Taylor and Weissbach, 1973)], (*c*) N_2O treatment [N_2O inhibits 5-CH_3-H_4PteGlu-homocysteine transmethylase by interacting with vitamin B_{12} (Scott *et al.*, 1981)], or (*d*) dietary folate deficiency (Rabinowitz and Tabor, 1958), result in the accumulation of formiminoglutamate from histidine. Situations (*a*)–(*c*) would be expected to be associated with the inability to utilize added 5-CH_3-H_4PteGlu as a source of folate coenzymes. This is an important point because 5-CH_3-H_4PteGlu is the predominant form of folate in serum (Herbert and Das, 1976). Experiments with vitamin B_{12}-deficient cultured L1210 murine leukemia cells yield results consistent with these considerations (Fujii *et al.*, 1982). The vitamin B_{12}-deficient cells accumulate 5-CH_3-H_4PteGlu when provided with PteGlu or 5-CHO-H_4PteGlu, and this accumulation is abolished on adding vitamin B_{12} to the cells.

The accumulation of 5-CH_3-H_4PteGlu at the expense of other folate compounds is usually termed the *methyl trap* (Noronha and Silverman, 1962; Nair and Noronha, 1983) and has been discussed extensively in relation to the megaloblastic anemias of folate and vitamin B_{12} deficiencies (Beck, 1975; Herbert, 1978; Scott and Weir, 1981) as well as the degeneration of the spinal cord associated with severe vitamin B_{12} deficiency and with N_2O treatment (Scott *et al.*, 1981). The methyl trap hypothesis adequately explains the results obtained in the rat liver (Krebs *et al.*, 1976) and L1210 (Fujii *et al.*, 1982) systems mentioned previously as well as the fact that methionine prevents the degeneration of the spinal cord induced by N_2O in monkeys (Scott *et al.*, 1981). However, this hypothesis does not explain the observation that methionine administration to patients with megaloblastic anemia due to vitamin B_{12} deficiency shows aggravated megaloblastosis in the bone marrow at the same time that urinary excretion of formiminoglutamate is diminished (Herbert and Das, 1976). Thus, although the folate deficiency is alleviated as judged by diminished formiminoglutamate excretion, this does not suffice to prevent megaloblastosis.

Further discussion of the metabolic relationship between folate and vitamin B_{12} can be found in the symposium volume edited by Zagalak and Friedrich (1979) and in the review by Shane and Stokstad (1983). A summary of the regulation of the metabolism of single-carbon units is given in Table X.

D. Biosynthesis of Thymidylate

The enzymes serine hydroxymethyltransferase, thymidylate synthase, and dihydrofolate reductase catalyze the reactions of the thymidylate cycle (Fig. 5), which converts the methylene group of CH_2-H_4PteGlu to the methyl group of thymidylate. This reduction reaction is coupled to the oxidation of H_4PteGlu to

TABLE X

Regulation of Folate Enzymes

Enzyme	Source	Effector	Effect	References
5-Methyltetrahydrofolate-homocysteine methyltransferase	Pig liver, *E. coli*	Adenosylmethionine	Stimulation: 5-CH_3-H_4PteGlu is demethylated to H_4PteGlu, and methionine is formed	Mangum and Scrimgeour (1962), Taylor and Weissbach (1973)
Methylenetetrahydrofolate reductase	Rat liver	Adenosylmethionine	Inhibition: Synthesis of 5-CH_3-H_4PteGlu is diminished, CH_2-H_4PteGlu is spared for other pathways	Kutzbach and Stokstad (1971a), Krebs *et al.* (1976)
	Pig liver	H_2PteGlu	Inhibition: Synthesis of 5-CH_3-H_4-PteGlu is diminished; CH_2-H_4PteGlu is spared for other pathways	Matthews and Baugh (1980)
Serine hydroxymethyltransferase	Pig liver	5-CH_3-H_4PteGlu	Inhibition: Serine is shunted to pyruvate for oxidation to CO_2 or for gluconeogenesis	Schirch and Ropp (1967), Matthews *et al.* (1982)
10-Formyltetrahydrofolate dehydrogenase	Pig liver, rat liver	H_4PteGlu	Inhibition: Conserves single-carbon units by preventing their oxidation to CO_2	Kutzbach and Stokstad (1971b), Scrutton and Beis (1979)
10-Formyltetrahydrofolate dehydrogenase	Rat liver	10-CHO-H_4PteGlu	Disposal of excess single-carbon units: When the concentration of 10-CHO-H_4PteGlu rises, the CHO group can be converted to CO_2	Krebs *et al.* (1976), Scrutton and Beis (1979)

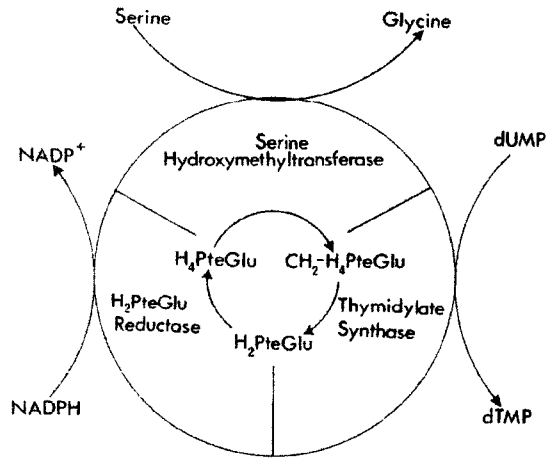

FIG. 5. The thymidylate cycle.

H_2PteGlu (Friedkin, 1973). The thymidylate cycle is crucial to the understanding of the mechanism of the cytotoxic action of methotrexate, trimethoprim, and 5-fluorouracil (Blakley, 1969; Danenberg, 1977; Hitchings and Roth, 1980; Heidelberger *et al.*, 1983; Hitchings, 1983). The cytotoxicity of the first two agents rests on their capacity to inhibit dihydrofolate reductase, which prevents the recycling of H_2PteGlu to H_4PteGlu and thus prevents synthesis of thymidylate. Thymidylate synthase is directly inhibited by 5-fluorodeoxyuridylate, a metabolite of fluorouracil.

Cells unable to synthesize thymidylate and devoid of an external source of thymidine undergo thymineless death (Cohen, 1971), apparently caused by the incorporation of uracil or fluorouracil into DNA followed by their excision, leading to fragmentation of DNA (Ingraham *et al.*, 1982; Herrick *et al.*, 1982; Ayusawa *et al.*, 1983b).

Blocking the thymidylate cycle is not the only mechanism by which methotrexate and fluorouracil cause cytotoxicity. Methotrexate can kill cells by blocking purine biosynthesis (Hryniuk *et al.*, 1975), and fluorouracil can be cytotoxic due to its incorporation into RNA (Glazer and Lloyd, 1982; Mandel, 1982; Dolnick and Pink, 1983). The small amount of fluorouracil incorporated into DNA (Kufe *et al.*, 1981; Cheng and Nakayama, 1983) may also be cytotoxic.

E. Biosynthesis of Purines

Formyltransferase reactions utilizing 10-CHO-H_4PteGlu are involved in the biosynthesis of formylglycinamide ribonucleotide and formylaminoimidazolecarboxamide ribonucleotide on the *de novo* pathway of purine biosynthesis (Smith *et al.*, 1981b; Buchanan, 1982) (Fig. 6).

Studies on the influence of methotrexate pretreatment on the metabolism of fluorouracil in L1210 cells illustrates the complex interrelationships between

purine and pyrimidine metabolism (Cadman *et al.*, 1981). Methotrexate inhibition of purine biosynthesis causes enhancement of 5-phosphoribosyl 1-pyrophosphate levels, which in turn increases the accumulation of fluorouracil ribonucleotides, leading to synergistic cell killing, apparently due to the increased incorporation of fluorouracil into RNA.

Methotrexate may inhibit purine biosynthesis by (*a*) blocking the conversion of H_2PteGlu formed in the thymidylate synthase reaction to H_4PteGlu, leading to a deficiency of formylation cofactors (Nixon *et al.*, 1973), or (*b*) direct inhibition of the formyltransferase reactions by methotrexate polyglutamates (Baggott 1983). Elevated levels of H_2PteGlu have been reported in methotrexate-inhibited L1210 cells (Nixon *et al.*, 1973; Jackson *et al.*, 1977), but no change in H_2PteGlu was seen in methotrexate-treated hamster ovary cells (McBurney and Whitmore, 1975).

Mutants of L1210 murine leukemia having impaired salvage are more sensitive to methotrexate toxicity *in vivo* than the parent line (Browman and Csullog 1981). These results support the view that one component of the cytotoxic action of methotrexate involves a purineless mechanism.

F. Role of the Mitochondrion

Both cytosolic and mitochondrial forms of serine hydroxymethyltransferase are known (Chasin *et al.*, 1974). In rabbit liver, the mitochondrial form is similar in structure and mechanism to the cytosolic form but differs in amino acid composition and in antigenicity and has a fourfold greater affinity for glycine

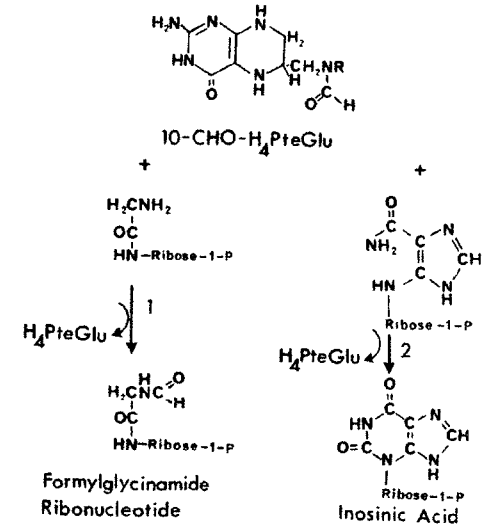

FIG. 6. Reactions catalyzed by (1) glycinamide ribonucleotide formyltransferase and (2) aminoimidazolecarboxamide ribonucleotide formyltransferase. Inosinic acid is the product because the enzyme also catalyzes ring closure. Mueller and Benkovic (1981).

(Schirch and Peterson, 1980). In Chinese hamster ovary cells, 25% of the enzyme activity is in the cytosol and 75% is in the mitochondria (Chasin *et al.*, 1974). A mutant lacking only the mitochondrial enzyme requires glycine for growth even though the cytosolic enzyme remains active. The mitochondrial enzyme regulates the supply of glycine available for growth (Pfendner and Pizer, 1980; Taylor and Hanna, 1982). Excess glycine can be cleaved by the glycine cleavage system in the mitochondria.

It has been proposed that the mitochondrial and cytosolic serine hydroxymethyltransferases are part of a shuttle system that serves to bring one-carbon units at the methylene level from the mitochondria to the cytosol (Cybulski and Fisher, 1976) (Fig. 7). CH_2-H_4PteGlu is formed in mitochondria from the oxidation of the methyl groups of dimethylglycine and sarcosine (Mitchell and Benevenga, 1976; Wittwer and Wagner, 1981), which are formed from betaine, an important source of methyl groups arising from dietary choline. The CH_2-H_4PteGlu then reacts with glycine to form serine, which is transported to the cytosol, where it can be reconverted to CH_2-H_4PteGlu and glycine. Some of the CH_2-H_4PteGlu generated in the mitochondria is used to produce formylmethionyl-tRNA, which is used in the initiation of mitochondrial protein synthesis (Galper and Darnell, 1969; Lucas-Lenard and Lipmann, 1971).

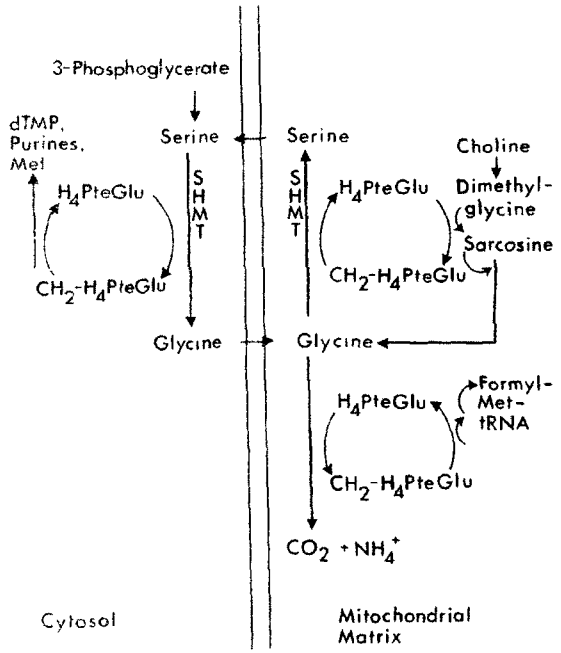

FIG. 7. Metabolic interrelationships between cytosolic and mitochondrial serine hydroxymethyltransferase (SHMT)

VII. Enzymes

A. Folate Enzymes of Wide Distribution

The enzymes discussed in this section (Table VIII) are numbered to correspond to the numbered reactions in the metabolic chart (Fig. 4). The Enzyme Commission numbers (Enzyme Nomenclature Committee, 1979) are given in Table VIII.

1. Dihydrofolate Reductase

a. General Properties. A great deal of literature has accumulated on this enzyme (Gready, 1980), and other chapters in this volume deal with enzyme structure and structure–activity relationships. Here we cover some biochemical topics of current interest.

Dihydrofolate reductase catalyzes the reactions

$$\text{PteGlu} + \text{NADPH} + \text{H}^+ \rightarrow 7,8\text{-H}_2\text{PteGlu} + \text{NADP}^+ \quad (1)$$

$$\text{H}_2\text{PteGlu} + \text{NADPH} + \text{H}^+ \rightarrow 5,6,7,8\text{-H}_4\text{PteGlu} + \text{NADP}^+ \quad (2)$$

In both reactions a hydrogen atom is transferred from the A side of the reduced nicotinamide (Pastore and Friedkin, 1962) to the same face of PteGlu and H_2PteGlu, that is, the face toward the viewer in Fig. 1 (Charlton *et al.*, 1979; Pastore *et al.*, 1980). The orientation of the hydrogen atoms can also be seen in Fig. 2a, where the hydrogen atoms added to C-6 and C-7 are to the right of the plane of the pteridine. It is possible that the 5,6-double bond is reduced in both reactions. In Eq. (1) the initial reduction could be at the 5,6-positions followed by a rearrangement of 7,8-H_2PteGlu.

The reversibility of Eq. (2) can be observed spectrophotometrically (Mathews and Huennekens, 1960). At pH 7.0 the equilibrium constant is 5.6×10^{-4}. Equation (1) is catalyzed much more slowly than Eq. (2) (Blakley, 1969), and it has a lower pH optimum. Little reduction of PteGlu is seen at pH 7.0. Nonetheless, dihydrofolate reductase is presumed to catalyze the conversion of PteGlu to H_4PteGlu *in vivo*.

Reaction of the single cysteine residue in chicken liver dihydrofolate reductase with organic mercurials, iodine, or tetrathionate causes up to a 10-fold increase in activity (Barbehenn and Kaufman, 1982). Physiological modifiers have not been found. No cysteine residues are found in the *Lactobacillus casei* or *Streptococcus faecium* enzymes. Both the chicken liver and *L. casei* enzymes are activated by NaCl (Dann *et al.*, 1976; Subramanian *et al.*, 1981). The mechanism of activation is not clear, but ^{35}Cl-NMR studies with the chicken liver preparation show that chloride ion and NADPH interact with the enzyme in a competitive manner (Subramanian *et al.*, 1981). It has been suggested that chloride may activate the enzyme by facilitating the release of products.

b. Substrate Specificity. Some folate analogs showing substrate activity with dihydrofolate reductase include H_2-homofolate (Plante *et al.*, 1967), H_2-11-

oxahomofolate (Nair *et al.*, 1980), H_2-11-thiohomofolate (Nair *et al.*, 1979), H_2Pte-pGlu (Kisliuk *et al.*, 1977), 7,8-H_2-biopterin (Kaufman, 1967), and H_2PteLys (Plante *et al.*, 1976). It was suggested some time ago that dihydrofolate reductase, which is sometimes present at high levels in methotrexate-resistant cell lines, might be utilized to catalyze the formation of a toxic H_4PteGlu analog *in vivo* (Plante *et al.*, 1967; Friedkin *et al.*, 1971). Such a situation has not yet been documented.

Dihydrofolate reductase catalyzes the reduction of H_2Pte polyglutamates (Coward *et al.*, 1974). With the human enzyme, decreased K_m values are seen with longer glutamate chains; however, with the L1210 enzyme little change is seen. $H_2PteGlu_6$ is a substrate for *L. casei* dihydrofolate reductase (Kisliuk *et al.*, 1974).

c. Subunit Structure. Most dihydrofolate reductases are monomers having molecular weights between 18,000 and 22,000. However, two types of dihydrofolate reductase coded by *E. coli* plasmids that confer resistance to trimethoprim are dimers or tetramers. Type I contains two subunits, each of M_r 18,000. Type II contains four subunits, each of M_r 8500. Type II enzyme does not contain the conserved amino acid residues found in the vast majority of enzymes that catalyze this reaction (Smith *et al.*, 1979; Fling and Elwell, 1980). The dihydrofolate reductase produced by coliphage T4 is a dimer with subunits of M_r 23,800 and thus resembles the plasmid type I enzyme (Purohit *et al.*, 1981). The T4 enzyme is less sensitive to trimethoprim inhibition than most bacterial dihydrofolate reductases.

In *Crithidia fasciculata, Plasmodium berghei,* and many other protozoans, dihydrofolate reductase shares a bifunctional peptide M_r 56,700 with thymidylate synthase (Ferone and Roland, 1980; Coderre *et al.*, 1983). In *C. fasciculata* this bifunctional protein occurs in aggregates of M_r 100,000–200,000.

An enzymatically inactive protein of M_r 41,000 that reacts with antibodies to purified calf liver dihydrofolate reductase has been found in human leukocytes (Rothenberg and Iqbal, 1982). Its relation to active dihydrofolate reductase is not known. Its concentration is much higher than that of active dihydrofolate reductase.

d. Cellular Distribution. Dihydrofolate reductase is reported to be in the cytoplasm of animal cells (Wang *et al.*, 1967; Blakley, 1969). Some studies suggest, however, that in Chinese hamster embryo fibroblast cells, six enzymes associated with DNA precursor metabolism, including dihydrofolate reductase, are associated with the nucleus during the S-phase of cell growth (Reddy and Pardee, 1980; Noguchi *et al.*, 1983).

Although dihydrofolate reductase is often associated with growing tissue, it is also present in brain (Spector *et al.*, 1977).

In vitro studies with *L. casei* dihydrofolate reductase show that it binds to *L. casei* DNA containing the dihydrofolate reductase gene, suggesting that the enzyme may regulate its own synthesis (Gronenborn and Clore, 1983).

e. Crystallographic Studies. The structures of three dihydrofolate reductases have been solved by X-ray crystallography. They are (*a*) the methotrexate–enzyme complex from *E. coli* (Matthews *et al.*, 1977), (*b*) the NADPH– methotrexate–enzyme complex from *L. casei* (Matthews *et al.*, 1978; Bolin *et al.*, 1982; Filman *et al.*, 1982), and (*c*) the NADPH–2,4-diamino-5,6-dihydro-6,6-dimethyl-5-(4′-methoxyphenyl)-*s*-triazine–enzyme complex from chicken liver (Volz *et al.*, 1982). From the wealth of useful information accrued from these studies, an outstanding fact is that methotrexate binds to the enzyme with its pteridine ring rotated 180° from that of the substrate H_2PteGlu. This possibility was suggested by Matthews *et al.* (1978) for the *L. casei* system and was proved to be the case when it was found that the absolute configuration of biological H_4PteGlu derivatives is opposite to that predicted from the NADPH– methotrexate– enzyme model (Fontecilla-Camps *et al.*, 1979). It is probable that Asp-26, the likely proton donor to the pteridine ring in the enzyme-catalyzed reaction, interacts with N-3 of H_2PteGlu rather than with the N-1 position, as is the case with methotrexate (Bolin *et al.*, 1982).

f. NMR Studies. The extensive and detailed NMR studies on dihydrofolate reductase and its interaction with ligands have been reviewed (Matthews, 1979; Blakley, 1981; Cohn and Reed, 1982; Roberts, 1983). Generally, the conformational mobility of the protein is decreased by ligand binding. The chemical shifts observed in hydrogen and carbon in histidine (Poe *et al.*, 1979a; Gronenborn *et al.*, 1981), methionine (Blakley *et al.*, 1978), arginine (Cocco *et al.*, 1977), and tryptophan (Groff *et al.*, 1981) residues on ligand binding are in accord with the structure of the dihydrofolate reductase involved. Some of the specific points elaborated by NMR studies are as follows. (*a*) ^{13}C-NMR–pH titration studies with the *S. faecium* enzyme show that the association constant for the binding of methotrexate is sufficiently increased when protonation of N-1 occurs to account for the increased binding of methotrexate as compared with folate (Cocco *et al.*, 1981), and (*b*) the 2′-P of NADPH binds to the *L. casei* enzyme as a dianion (Hyde *et al.*, 1980). The signal for this atom bound to the *E. coli* enzyme is smaller, indicating that the conformation of NADPH differs in the two enzyme complexes (Feeney *et al.*, 1977).

Even with the extensive structural information available for dihydrofolate reductase, it is often difficult to assign the NMR bands for individual amino acid residues. Studies in which [γ-^{13}C]tryptophan (Fig. 8a) was incorporated into the *S. faecium* and *L. casei* enzymes illustrate this point. Each of these enzymes has four tryptophan residues. In the case of the *S. faecium* enzyme, four NMR bands are seen, two of which are altered by ligand binding (Groff *et al.*, 1981). It is not possible to assign the NMR bands to specific residues with certainty. Similar

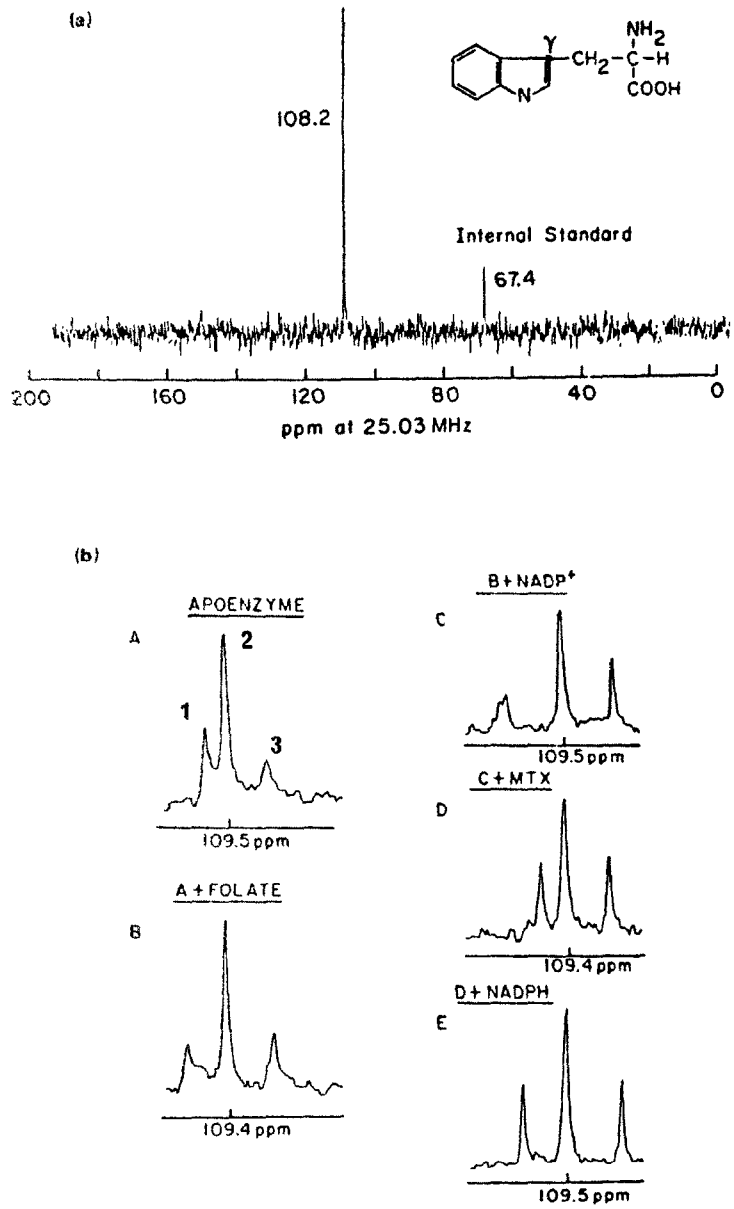

FIG. 8. ^{13}C-NMR spectra of [γ-^{13}C]tryptophan (a) and *Lactobacillus casei* dihydrofolate reductase containing [γ-^{13}C]tryptophan (b). The internal standard is tetramethylsilane. The ligands were added to the apoenzyme (A) in the order indicated. Because methotrexate (MTX) and NADPH displace folate and NADP, respectively, from the enzyme, the spectra shown are (B) enzyme–folate, (C) enzyme–NADP–folate, (D) enzyme–NADP–methotrexate, and (E) enzyme– NADPH– methotrexate. The ligand/enzyme concentration ratios were folate, 1.2; NADP, 2; methotrexate, 1.2; and NADPH, 1.2.

difficulty is encountered with the *L. casei* enzyme containing [γ-^{13}C]tryptophan in positions 5, 21, 133, and 158 (Pastore *et al.*, 1981; E. J. Pastore, L. T. Plante, R. L. Kisliuk, J. M. Wright, D. Strumpf, and N. O. Kaplan, unpublished). In this instance only three NMR bands are seen, one of which (band 2) has double amplitude (Fig. 8b). This large band, which is close to that seen in free tryptophan, is unaltered by PteGlu, NADP, NADPH, or methotrexate binding in binary or ternary complexes and would appear to belong to residues 133 and 158, which are remote from the active site and in close proximity to one another (Filman *et al.*, 1982). However, refinement of the X-ray data has shown that the local environments of the γ-carbons of these two residues in the crystal are different; that of residue 133 is at the surface and solvent accessible (eight H_2O within a 6-Å radius), whereas that of residue 158 is buried and tightly packed by various side chains. The observed magnetic equivalence of the γ-carbons of Trp-133 and Trp-158, despite their environmental differences in the crystal, suggest that the solution and crystal structures of the enzyme are not the same or that the resonances are incorrectly assigned. Band 3 at 107.5 ppm in the apoenzyme (Fig. 8A) most likely represents Trp-5 because its chemical shift is upfield from tryptophan in solution, consistent with the fact that the side chain of Trp-5 is directed to a hydrophobic pocket, which would be expected to result in the increased shielding. Ligand binding leads to a sharpening of this band, indicating decreased mobility. The peptide backbone at Trp-5 is in contact with the pteridine ring of methotrexate in the methotrexate–NADPH-enzyme complex (Bolin *et al.*, 1982). Band 1 (Fig. 8A) at 110.4 ppm most likely arises from Trp-21, which is a contact residue for both methotrexate and NADPH. This residue is conserved in most dihydrofolate reductases, which is not the case for residues 5, 133, and 158 (Freisheim *et al.*, 1979). Mobility of this residue is indicated by the doublet observed in the PteGlu–enzyme binary complex (Fig. 8B) and in the PteGlu–NADPH–enzyme–ternary complex (Fig. 8C) but is not present in the complexes with methotrexate.

g. Calorimetry. Major differences are seen in the binding of pyridine nucleotides and folates to the chicken liver enzyme (Subramanian and Kaufman, 1978). NADPH and NADP show small negative enthalpies and large positive entropies, whereas the binding of folate and methotrexate yields large negative enthalpies and small negative entropies. The enthalpy of methotrexate binding demonstrates that proton transfer is associated with binding, which is not found with PteGlu or H_2PteGlu.

h. Laser Raman Spectroscopy. Studies with the *L. casei* enzyme show that the 1685 cm^{-1} band assigned to the carboxamide of NADPH persists in the NADPH–enzyme binary complex but is absent from the NADPH– methotrexate– enzyme ternary complex (Dwivedi *et al.*, 1981). This is ascribed to stabilization of the polarized form of the carboximide by hydrogen bonding to the NH

and CO groups of Ala-6 and Ile-13 of the peptide backbone on ternary complex formation.

Ozaki *et al.* (1981) showed that there are marked spectral differences at the 1300–1350 cm^{-1} region between free and enzyme-bound (*L. casei*) methotrexate, which suggest changes at the benzoylamide bond. ^{13}C-NMR studies also indicate changes at this bond (Pastore *et al.*, 1979). Benzoyl[^{13}C]carbonyl-labeled folate and aminopterin show strong shielding in the enzyme-bound forms attributed to the proximity of the [^{13}C]carbonyl to Phe-49. *Streptococcus faecium* dihydrofolate reductase lacks an aromatic amino acid in the corresponding position, and shielding is not observed with the enzyme bound benzoyl[^{13}C]carbonyl-labeled folate or aminopterin (Pastore *et al.*, 1979).

2. *Serine Hydroxymethyltransferase*

a. General Properties. This enzyme catalyzes the interconversion of glycine and serine:

$$\text{Ser} + H_4\text{PteGlu} \leftrightarrow CH_2\text{-}H_4\text{PteGlu} + \text{Gly} \qquad (3)$$

The reaction is reversible and has an equilibrium constant of 10 (Rader and Huennekens, 1973). Pure enzyme has been obtained from rabbit (Schirch and Mason, 1963), pig (Matthews *et al.*, 1982), beef (Jones and Priest, 1976), and lamb liver (Ulevich and Kallen, 1977). The role of the cytosolic and mitochondrial forms of this enzyme in the generation of single-carbon units is discussed in Section VI,F. Schirch (1982) has reviewed research on serine hydroxymethyltransferase with emphasis on its structure and mechanism.

The liver enzymes are tetramers containing four identical subunits of M_r 55,000. Each subunit contains a molecule of pyridoxal phosphate. Cooperative interactions among the subunits on binding pyridoxal phosphate or H_4PteGlu have not been demonstrated (Schirch and Quashnock, 1981; Quashnock *et al.*, 1983). H_4PteGlu has at least two roles in the reaction (Schirch, 1982). It enhances the addition and removal of CH_2O from the active site, and it facilitates the removal of the 2*s* proton of glycine to a base on the enzyme (Fig. 9). However, pure enzyme catalyzes serine formation from CH_2O and glycine in the absence of H_4PteGlu. Pyridoxal phosphate is required for activity; one molecule is present on each subunit of the enzyme bound as a Schiff base to an ε-amino group of lysine. Pyridoxal phosphate can be removed from the enzyme by treatment with L-Cys or D-Ala.

Serine hydroxymethyltransferase forms ternary complexes with glycine and CH_3-H_4PteGlu or with glycine and H_4PteGlu, which have an absorption peak at 497 nm (Schirch, 1982). This permits spectrophotometric titration of the interaction of ligands with the enzyme and the determination of dissociation constants. The absorption peak is due to a quinonoid form of pyridoxal phosphate (Fig. 9). Increasing the polyglutamate chain length of CH_3-H_4PteGlu enhances the formation of the inactive glycine–5-CH_3-H_4PteGlu–enzyme complex, which *in vivo* would diminish the formation of single-carbon units from serine, as discussed in Section VI,C (Matthews *et al.*, 1982).

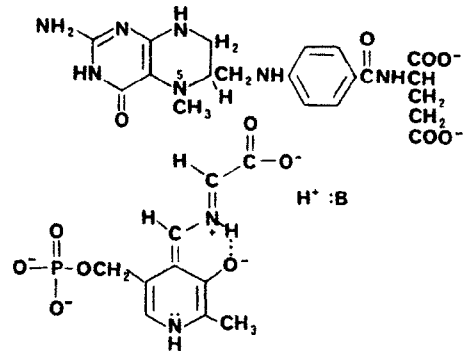

FIG. 9. Enzymatically inactive complex of serine hydroxymethyltransferase, 5-methyl tetrahydrofolate, glycine, and the quinonoid form of pyridoxal phosphate (λ_{max} = 497 nm) in which the 2*s* proton of glycine is transferred to a basic group on the protein.

b. Substrate Specificity. Some of the reactions catalyzed by serine hydroxymethyltransferase are listed in Table XI (Schirch, 1982). Four types of reaction are shown: aldol cleavage (reactions 1–3), transamination (reaction 4), defluorination (reaction 5), and decarboxylation (reaction 6). Only reaction 1 has a requirement for H_4PteGlu. Reactions 2 and 3 show that the enzyme is not specific as to the stereochemical configuration at the β-carbon of the amino acid in that it catalyzes the cleavage of both threonine and allothreonine. Isotope studies demonstrate that inversion of the configuration of the hydrogen atoms on the CH_2 group occurs during the conversion of serine to glycineto the extent of 24% (Tatum *et al.*, 1977b). Similar partial stereospecificity is found during the conversion of formate to serine by rat liver slices (Biellmann and Schuber,

TABLE XI

SOME REACTIONS CATALYZED BY SERINE HYDROXYMETHYLTRANSFERASE[a]

1. L-Serine + H_4PteGlu → glycine + CH_2-H_4PteGlu
2. L-Threonine → glycine + acetaldehyde
3. L-Allothreonine → glycine + acetaldehyde
4. D-Alanine + pyridoxal phosphate → pyruvate + pyridoxamine phosphate
5. β-D-Fluoroalanine → HF + pyruvate + NH_4^+
6. Aminomalonate → glycine + CO_2

[a]From Schirch (1982) and Wang *et al* (1981).

1967). When glycine and acetaldehyde are incubated with the enzyme, the products are 98% allothreonine and 2% threonine (Schirch, 1982).

CH_2-H_4PteGlu$_n$ has a much higher affinity for the pig liver enzyme than does H_4PteGlu (Matthews *et al.*, 1982).

c. *Inhibitors.* Serine hydroxymethyltransferase is a potential site for the cytotoxic action of chemotherapeutic agents because its inactivation would be expected to lead to lower thymidylate formation due to diminished levels of CH_2-H_4PteGlu. D-Cycloserine, which inhibits the enzyme, is reported to inhibit the growth of Friend leukemia cells in mice (Bukin *et al.*, 1979; Bukin and Draudin-Krylenko, 1980). Tetrahydrohomofolate inhibits the serine hydroxymethyltransferase of L1210 cells (Scanlon *et al.*, 1981), but whether this inhibition is responsible for the cytotoxic effect of the compound is uncertain (Kisliuk, 1982).

β-D-Fluoroalanine is an interesting inhibitor of serine hydroxymethyltransferase (Wang *et al.*, 1981). The enzyme catalyzes two reactions with this compound. The first involves its conversion to pyruvate, hydrogen fluoride, and ammonia and is harmless (Table XI). However, once in 50 turnovers, a cysteine residue near the active site becomes alkylated by a three-carbon residue from the substrate and the enzyme is inactivated. The inactivation reaction is enhanced by adding H_4PteGlu.

3. *Glycine Cleavage System*

Glycine is reversibly cleaved to CO_2, NH_3, and CH_2-H_4PteGlu by the glycine cleavage system (Fig. 10), which has been demonstrated in animals (Hiraga and Kikuchi, 1980a), plants (Cossins and Sinha, 1966), and bacteria (Ogur *et al.*, 1977). The components of the system have been purified from chicken liver mitochondria (Hiraga and Kikuchi, 1980a,b) and from *Peptococcus glycinophilus* (Robinson *et al.*, 1973). Both systems consist of four proteins, which play a similar role in each system. The chicken liver proteins are designated P, H, T, and L (Fig. 10).

The P-protein, or glycine decarboxylase, consists of two identical subunits of molecular weight 100,000, each containing a molecule of pyridoxal phosphate. Alone, this protein has very low activity but can slowly catalyze the formation of CH_3NH_2 from glycine. The activity of P-protein is greatly stimulated by H-protein (M_r 14,000), which contains lipoic acid. Lipoic acid alone can stimulate P-protein activity. The P-protein and H-protein interact to form a complex, which can be demonstrated by gel filtration or sucrose density gradient centrifugation. One molecule of H-protein binds to each subunit of P-protein. The complex catalyzes the conversion of glycine to CO_2, NH_3, and CH_2O.

The T-protein (M_r 40,000) requires H_4PteGlu for its activity and catalyzes the degradation of an enzyme-bound —CH_2NH_2 moiety to CH_2-H_4PteGlu and NH_3 (Okamura-Ikeda *et al.*, 1982). This protein is basic (pI = 9.8) and forms a 1:1 complex with the acidic H-protein (pI = 4.0). The T-protein of rat liver

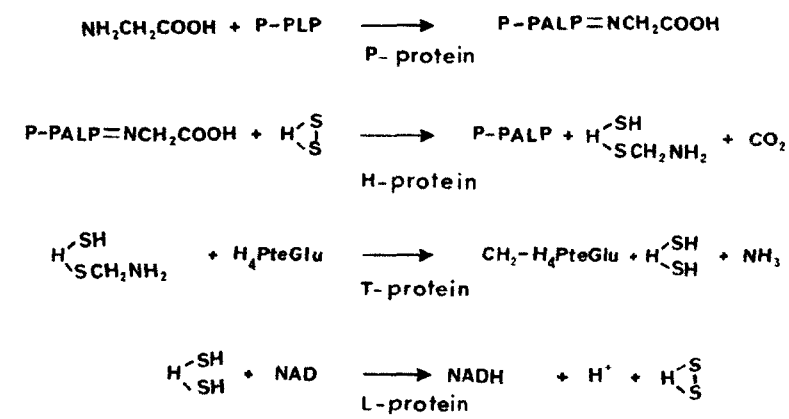

FIG. 10. Reactions of the glycine cleavage system (PLP, pyridoxal phosphate, PALP, pyridoxamine phosphate).

mitochondria has a molecular weight of 33,000 and is less stable than the chicken liver mitochondrial enzyme (Motokawa and Kikuchi, 1974). The L-protein is an NAD-dependent flavoprotein that catalyzes the dehydrogenation of reduced H-protein (Motokawa and Kikuchi, 1974).

Likely functions for the glycine cleavage system are the metabolism of excess dietary glycine or glycine arising from serine and choline and the provision of a source of formyl groups for the formation of formylmethionyl-tRNA, which is required for the initiation of protein synthesis in mitochondria (Fig. 7). It has been shown that glycine metabolism is sensitive to the oxidation state of mitochondria (Hampson *et al.*, 1983). Metabolism was maximal in the uncoupled state and low when respiration was inhibited.

In humans afflicted with nonketotic hyperglycinemia, the glycine cleavage system is defective, leading to high glycine levels, which interfere with nerve function (Stanbury *et al.*, 1983).

4. *Thymidylate Synthase*

a. General Properties. Thymidylate synthase, dihydrofolate reductase, and serine hydroxymethyltransferase are components of a system that catalyzes the formation of dTMP from dUMP (Fig. 5). Thymidylate synthase catalyzes the reductive methylation of dUMP (Friedkin, 1973), utilizing CH_2-H_4PteGlu as the source of the single-carbon unit as well as the reductant:

$$\text{dUMP} + \text{CH}_2\text{-H}_4\text{PteGlu} \rightarrow \text{dTMP} + \text{H}_2\text{PteGlu} \quad (4)$$

Dihydrofolate reductase and serine hydroxymethyltransferase catalyze the regeneration of CH_2-H_4PteGlu from H_2PteGlu.

The hydrogen of C-6 of CH_2-H_4PteGlu stereospecifically reduces the meth-

hydroxymethyl group to a methyl group (Pastore and Friedkin, 1962; Tatum *et al.*, 1977a). The reaction is irreversible, the equilibrium constant being too low to measure (Rothman *et al.*, 1973).

The enzyme catalyzes the exchange of the hydrogen on C-5 of the dUMP with the protons of water (Lomax and Greenberg, 1967) as well as the dehalogenation of 5-iodo- and 5-bromo-dUMP (Garrett *et al.*, 1979).

A list of some sources of purified thymidylate synthase along with their M_r and K_m values are given in Table XII. In *L. casei* (Crusberg *et al.*, 1970) and *S. faecium* (Albrecht *et al.*, 1966) the levels of both thymidylate synthase and dihydrofolate reductase are enhanced in methotrexate-resistant strains. In *Crithidia fasciculata* and other parasitic protozoans, thymidylate synthase and dihydrofolate reductase are found associated with the same polypeptide chain (Ferone and Roland, 1980; Coderre *et al.*, 1983). Recombinant DNA techniques have been used to enhance levels of the *E. coli* and coliphage T4 thymidylate synthases to a great extent (Belfort *et al.*, 1983a,b).

TABLE XII

SOURCES OF PURIFIED THYMIDYLATE SYNTHASE

Source	K_m (μM) CH_2-H_4PteGlu	K_m (μM) dUMP	Dimer M_r	References
Methotrexate-resistant *Lactobacillus casei*	24[a]	5[c]	73,176	Leary and Kisliuk (1971), Galivan *et al.* (1975), Dunlap (1978), Maley *et al.* (1979a), Kisliuk *et al.* (1981)
Methotrexate-resistant *Streptococcus faecium*	30[a]	8	72,000	Rao and Kisliuk (1983)
Escherichia coli	14[a]	10	64,000	Haertle *et al.* (1979), Belfort *et al.* (1983b)
Coliphage T4	20[a]	6	64,000	Belfort *et al.* (1983a)
Saccharomyces cerevisiae	70[a]	5	65,000	Bisson and Thorner (1981)
Ehrlich ascites carcinoma	43[b]	6	69,000	Jastreboff *et al.* (1982)
Chick embryo	14[b]	8	58,000	Lorenson *et al.* (1967)
L1210 cells			75,000	Rode *et al.* (1979)
HeLa cells	31[b]	2	72,000	Rode *et al.* (1980)
Human leukemic cells	31[b]	2	66,000	Dolnick and Cheng (1977), Lockshin *et al.* (1979)
Calf thymus	16[b]	9	77,000	Horinishi and Greenberg (1972), Dwivedi *et al.* (1983b)
Crithidia fasciculata	400[c]	3	107,000	Ferone and Roland (1980)

[a] Natural diastereoisomer at C-6.
[b] Mixture of diastereoisomers at C-6.
[c] With dihydrofolate reductase on the same protein.

The amino acid sequences of *L. casei* (Maley *et al.*, 1979a) and *E. coli* (Belfort *et al.*, 1983c) thymidylate synthases are known.

b. Substrate Specificity. Congeners of dUMP in which either of the 2′-hydrogen atoms is replaced with a hydroxyl group or fluorine atom show detectable substrate activity (Table XIII). These four analogs have not been tested under comparable conditions. The conformation of the pentose proposed as most favorable for substrate activity is 2′-endo (*S*) (Haertle *et al.*, 1979) (Fig. 11), but the evidence for this view is incomplete (Birnbaum *et al.*, 1982).

^{31}P-NMR and calorimetric studies show that dUMP binds to the *L. casei* enzyme with the phosphate in the dianionic form (Beckage *et al.*, 1979; Beaudette *et al.*, 1980). Compounds incapable of forming a dianion, such as the methyl phosphate derivative of dUMP, are not active as substrates for the *E. coli* enzyme (Holy and Votruba, 1974). A likely site for the interaction of the phosphate is an arginine residue (Belfort *et al.*, 1980).

With the *L. casei* enzyme, 12- to 20-fold lower K_m values are seen with $H_4PteGlu_{3-7}$ as cofactors as compared with $H_4PteGlu$ (K_m = 24 μM) (Kisliuk *et al.*, 1981). However, with the calf thymus enzyme, $H_4PteGlu$, $H_4PteGlu_3$, and $H_4PteGlu_7$ all have K_m values near 15 μM (Dwivedi *et al.*, 1983a).

Pteroyl polyglutamates have a higher affinity for thymidylate synthase than the

TABLE XIII

ALTERNATIVE SUBSTRATES FOR THYMIDYLATE SYNTHASE

Compound	Enzyme source	References
Pyrimidine nucleotides		
UMP	Chick embryo	Lorenson *et al.* (1967)
	Lactobacillus casei	Dunlap *et al.* (1971)
Ara-UMP	Coliphage T2	Pizer and Cohen (1960)
	Escherichia coli K12	Wohlrab *et al.* (1978)
2′-Deoxy-2′-fluororibouridylate	*E. coli* K12	Wohlrab *et al.* (1978), Haertle *et al.* (1979)
2′-Deoxy-2′-fluoro-Ara-uridylate	*L. casei*	Braun *et al.* (1982)
4-Thio-2′-dUMP	*L. casei*	Kalman *et al.* (1973)
Folates		
5,11-CH_2-H_4-Homofolate	*L casei*	Crusberg *et al.* (1970)
	Streptococcus faecium	Kisliuk and Gaumont (1970)
	Mouse reticulocytes	Reid and Friedkin (1973)
	HeLa cells	Taylor and Hanna (1974)
	CHO cells	Taylor and Hanna (1974)
	L1210 cells	Scanlon *et al.* (1981)
CH_2-H_4PteLys and CH_2-H_4Pte(di-α-lysyl)-Lys	*E. coli*	Plante *et al.* (1976)

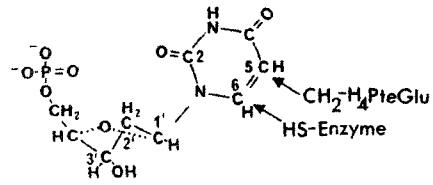

FIG. 11. Interaction of dUMP with methylene tetrahydrofolate and thymidylate synthase. The nucleotide is shown in its syn-conformation, and the conformation of the furanose ring is 2′-endo (*S*).

corresponding monoglutamate forms, as evidenced by the enhanced inhibitory potency of polyglutamate derivatives of PteGlu and methotrexate (Table XIV).

H_4-Homofolate, having an additional methylene group between C-9 and N-10, replaces H_4PteGlu as a cofactor for many thymidylate synthases (Table XIII). H_4-Homofolate inhibits *E. coli* thymidylate synthase (Goodman *et al.*, 1964) but becomes a substrate if two γ-glutamate residues are added (Friedkin *et al.*, 1971). H_4-Homofolate derivatives are active against methotrexate-resistant L1210 cells (Kisliuk, 1982). H_4PteLys and H_4PteLys$_n$ are substrates for *E. coli* thymidylate synthase (Plante *et al.*, 1976). Thus, changing the polarity from the negative glutamate residue of H_4PteGlu to the positive lysine residues does not abolish substrate activity. Indirect evidence suggests that the enzyme tolerates a cofactor with an extra HN group inserted between the *p*-aminobenzoate carbonyl and its phenyl ring (Martinelli *et al.*, 1979). H_4Pte-D-Glu does not serve as a cofactor for *L. casei* thymidylate synthase (Kisliuk *et al.*, 1977).

c. Substrate Interactions. Circular dichroism studies suggest that the conformation of dUMP changes from anti to syn on binding to *L. casei* thymidylate synthase (Leary *et al.*, 1975); that is, the oxygen atom on C-2 of the pyrimidine ring, which is pointed away from the furanose ring when the nucleotide is in solution, turns toward it on binding with the enzymes (Fig. 11). ^{19}F-NMR studies on the noncovalent binary complex of 5-fluoro-2′-deoxyuridylic acid (FdUMP) and *L. casei* thymidylate synthase are consistent with the proposed syn-conformation for the bound nucleotide (Lewis *et al.*, 1980).

H_4PteGlu does not bind to the *L. casei* enzyme in the absence of dUMP, but H_4PteGlu$_4$ does so (Galivan *et al.*, 1976). Inactivation of the nucleotide binding site with iodoacetate does not prevent the binding of CH_2-H_4PteGlu and, conversely, inactivation of the folate site by removal of the carboxy terminal valine residue (Aull *et al.*, 1974) does not prevent nucleotide binding.

The effect of Mg^{2+} on thymidylate synthase activity is variable. It stimulates the enzymes from *E. coli* (Wahba and Friedkin, 1962), *S. faecium* (Blakley and McDougall, 1962), *Diplococcus pneumoniae* (McCuen and Sirotnak, 1975), coliphage T2 (Maley *et al.*, 1979b), *L. casei* (Dunlap *et al.*, 1971), and Ehrlich ascites cells (Jastreboff *et al.*, 1982) but is without effect on thymidylate synthases from calf thymus (Horinishi and Greenberg, 1972), chick embryo (Lorenson *et al.*, 1967), L1210 cells (Livingston *et al.*, 1968), or human leukemia cells (Lockshin *et al.*, 1979). Magnesium ion inhibits enzyme activity in extracts of *Aedes aegypti* (Jaffe and Chrin, 1979). In all of these instances, CH_2-H_4PteGlu was the substrate. With the *L. casei* enzyme Mg^{2+} activation is not seen if CH_2-H_4PteGlu$_3$ is substituted for CH_2-H_4PteGlu (Kisliuk *et al.*, 1981).

TABLE XIV

INHIBITORS OF THYMIDYLATE SYNTHASE

Compound	Inhibitory potency	Enzyme source	References
A. Pyrimidine nucleotides			
1. 5-Fluoro-dUMP	0.01[a]	*Lactobacillus casei*	Heidelberger *et al.* (1983)
2. Phosphonate analog of 5-fluoro-dUMP (5′-O replaced by CH_2)	0.06[b]	T2	Montgomery *et al.* (1979)
3. 5-Trifluoromethyl-dUMP	0.04[a]	*L. casei*	Heidelberger (1975), Santi (1980)
4. 5-Mercapto-dUMP	0.04[a]	*Escherichia coli*	Kalman and Bardos (1970)
5. 5-Ethynyl-dUMP	2.7[a]	*L. casei*	Danenberg *et al.* (1981), Barr *et al.* (1983)
6. 5-Nitro-dUMP	0.03[a]	*L. casei*	Washtien and Santi (1979)
7. 5-Formyl-dUMP	0.02[a]	*L. casei*	Balzarini *et al.* (1982)[c]
B. Folates			
1. 5,8-Dideaza-10-CH_3-folate	0.1[b]	*E. coli*	Bird *et al.* (1970)
2. 5,8-Dideaza-10-propargyl folate	0.005[b]	L1210	Jones *et al.* (1981)
	0.04[b]	*L. casei*	Nair *et al.* (1983)
3. 5,8-Dideaza-10-cyanomethyl folate	0.7[b]	*L. casei*	Nair *et al.* (1983)
4. PteGlu	150[b]	*L. casei*	Kisliuk *et al.* (1974)
$PteGlu_6$	0.6[b]		
$H_2PteGlu_6$	3.0[b]		
5. Methotrexate	20[b]	Calf thymus	Dwivedi *et al.* (1983a)
Methotrexate-Glu_6 (2,4-diamino-10-CH_3-$PteGlu_7$)	1.0[b]		

[a] K_i (micromolar).
[b] I_{50} (micromolar).
[c] Contains an extensive list of inhibitors and K_i values

d. Subunit Structure. *Lactobacillus casei* thymidylate synthase is a dimer with subunits of identical amino acid sequence (Maley *et al.*, 1979a). When a single ligand such as dUMP, FdUMP, or iodoacetamide is incubated with the

enzyme, only one of the two subunits reacts (Leary *et al.*, 1975; Galivan *et al.*, 1976; Beaudette *et al.*, 1980). It is postulated that, in the thymidylate synthase dimer, the asymmetric arrangement of subunits is such that the active site on one subunit is open and that on the other is closed (Beaudette *et al.*, 1977; Danenberg and Danenberg, 1979). Single ligands cannot open the second site, but this site opens when dUMP or FdUMP is present together with CH_2-H_4PteGlu.

With the *D. pneumoniae* and *E. coli* enzymes, the dissociated monomers are catalytically active (McCuen and Sirotnak, 1975). This has not yet been demonstrated to be the case with other thymidylate synthases. The calf thymus enzyme is not dissociated into dimers by heating with sodium dodecylsulfate under reducing conditions. However, peptide maps indicate that the enzyme is a dimer with each subunit having the same amino acid sequence (Dwivedi *et al.*, 1983b).

e. Cellular Distribution. In bacteriophage T4 (Chiu *et al.*, 1982; Allen *et al.*, 1983) and hamster fibroblasts (Reddy, 1982; Noguchi *et al.*, 1983), thymidylate synthase is associated with an enzyme complex that produces and channels deoxynucleotides for DNA synthesis. Thymidylate synthase is also associated with rapidly sedimenting material in extracts of mouse mammary carcinoma cells (Ayusawa *et al.*, 1983a) and *S. faecium* (Rao and Kisliuk, 1983). In *S. faecium* the enzyme is associated with RNA.

f. Interaction with Fluorodeoxyuridylate. The dUMP analog FdUMP is extensively used in studies of the mechanism of thymidylate synthase (Heidelberger *et al.*, 1983). When FdUMP is substituted for dUMP in the enzymatic reaction, the SH group of Cys-198 of the *L. casei* enzyme adds across the 5,6-double bond to form a covalent complex containing FdUMP, CH_2-H_4PteGlu, and enzyme (Bellisario *et al.*, 1976; Byrd *et al.*, 1978; Lewis *et al.*, 1981). From these studies as well as studies of the mechanism of the enzyme (Kalman, 1971; Santi, 1980; Barr *et al.*, 1983) it seems likely that an analogous series of reactions occurs with the substrate dUMP. The analog FdUMP is an inhibitor rather than a substrate because the C—F bond is not cleaved by the enzyme. The cellular level of CH_2-H_4PteGlu is an important determinant of fluorouracil toxicity (Ullman *et al.*, 1978; Houghton *et al.*, 1981; Yin *et al.*, 1983). The formation of the inactive ternary complex in cells treated with fluorouracil is enhanced by the addition of 5-CHO-H_4PteGlu (Waxman and Bruckner, 1982). Most cells are capable of converting fluorouracil to FdUMP and 5-CHO-H_4PteGlu to CH_2-H_4PteGlu. Fluorouracil can also inhibit cell growth due to its incorporation into RNA (Section VI,D).

Thymidylate synthase levels are enhanced in fluorodeoxyuridine-resistant mouse cells derived from neuroblastoma (Baskin *et al.*, 1975), hepatoma (Wilkinson *et al.*, 1977; Priest *et al.*, 1980b), and fibroblasts (Rossana *et al.*, 1982). A line of human lymphocytic leukemia cells resistant to fluorodeoxyuridine contains a thymidylate synthase with much lower affinity for fluorodeoxyuridylate (Bapat *et al.*, 1983).

g. Inhibitors of Thymidylate Synthase. A series of pyrimidine nucleotide inhibitors of thymidylate synthase is listed in Table XIV.

Compound 2, the phosphonate analog of FdUMP, was prepared with the expectation that the phosphonate group would not be as easily removed *in vivo* as compared with the corresponding phosphate. This compound is a potent inhibitor of thymidylate synthase and is moderately cytotoxic to Hep-2 cells in culture (I_{50} = 45 μM).

Compound 3,5-CF_3-dUMP is incorporated into viral DNA and is also a potent inhibitor of thymidylate synthase, which catalyzes labilization of the C—F bonds.

Compounds 4, 5, 6, and 7 are mechanism-based inhibitors of thymidylate synthase which, by analogy with FdUMP, probably act by forming a covalent bond between the 6-position of the pyrimidine and the sulfur atom of Cys-198 in the *L. casei* enzyme (Maley and Maley, 1981). 5-NO_2-dUMP is unique in that it readily forms a covalent bond with the enzyme in the absence of CH_2-H_4PteGlu. It has been shown that FdUMP can also form a covalent binary complex with *L. casei* thymidylate synthase in the absence of CH_2-H_4PteGlu (Ahmed *et al.*, 1983).

A series of folate analog inhibitors of thymidylate synthase is also shown in Table XIV. By far the most active is 5,8-dideaza-10-propargyl folate. Both folate and methotrexate show enhanced inhibition when γ-glutamate residues are added.

5. CH_2-H_4PteGlu Reductase

This enzyme catalyzes the reduction of CH_2-H_4PteGlu to 5-CH_3-H_4PteGlu utilizing enzyme-bound $FADH_2$ as the reductant. NADPH is the reductant for the enzyme-bound FAD (Daubner and Matthews, 1982). The reduction reaction is strongly favored (Katzen and Buchanan, 1965), but the enzyme can be assayed in the reverse direction if artificial electron acceptors such as menadione are included in the incubation (Donaldson and Keresztesy, 1962).

The enzyme has been obtained in pure form from pig liver (Daubner and Matthews, 1982) and has a subunit M_r of 75,000. The native enzyme is most likely a dimer. The K_m for CH_2-H_4PteGlu is 7 μM, but the value for CH_2-H_4PteGlu$_6$ is 0.1 μM (Matthews and Baugh, 1980). The K_m for NADPH with CH_2-H_4PteGlu as substrate is 16 μM, but with CH_2-H_4PteGlu$_6$ it is raised to 185 μM.

Adenosylmethionine is an allosteric inhibitor of the enzyme (I_{50} = 0.1 mM; Kutzbach and Stokstad, 1971a). H_2PteGlu$_6$ ($K_i = 1 \times 10^{-8}$ M; Matthews and Baugh, 1980) also inhibits the enzyme. These inhibitions are thought to play a role in the regulation of the metabolism of single-carbon units (Table X).

Humans lacking CH_2-H_4PteGlu reductase excrete homocystine in the urine due to their inability to remethylate homocysteine to methionine (Mudd *et al.*, 1972).

The kinetic mechanism of pig liver CH_2-H_4PteGlu reductase differs depending on whether short- or long-chain polyglutamate substrates are used. With CH_2-$H_4PteGlu_{1-3}$, the oxidation of enzyme-bound $FADH_2$ by CH_2-H_4PteGlu is rate limiting, whereas with $H_4PteGlu_{4-7}$ either product release or FAD reduction controls the rate of the overall reaction (Matthews and Baugh, 1980).

A likely chemical mechanism proposed for the reaction involves ring opening at N-10 to form the 5-iminium cation, which tautomerizes to quinonoid 5-CH_3-H_4PteGlu, which in turn is reduced to 5-CH_3-H_4PteGlu by $FADH_2$ (Fig. 12; Matthews, 1982). Important evidence in support of this proposal is that pig liver CH_2-H_4PteGlu reductase has quinonoid dihydropterin reductase activity (Matthews and Kaufman, 1980). Dihydropterin reductase is involved in reducing quinonoid dihydropterins formed in hydroxylation reactions, such as that catalyzed by phenylalanine hydroxylase, to tetrahydropterins. It is uncertain whether CH_2-H_4PteGlu reductase plays such a role *in vivo* (Matthews and Kaufman, 1980).

The methylene hydrogens of CH_2-H_4PteGlu are not exchanged with the medium during the course of the reaction (Kisliuk, 1963; Matthews, 1982).

c. 5-CH_3-H_4PteGlu-Homocysteine Methyltransferase*

Two types of enzyme are known to catalyze this reaction. One is found in plants and requires only substrates and Mg^{2+} for activity (Poston and Stadtman, 1975), and the other, found in animals, contains a reduced form of vitamin B_{12} as a cofactor and is primed by adenosylmethionine (Taylor and Weissbach, 1973; Huennekens *et al.*, 1975):

$$5\text{-}CH_3\text{-}H_4PteGlu + HSCH_2CH_2CHNH_2COOH \rightarrow CH_3SCH_2CHNH_2COOH + H_4PteGlu \quad (5)$$

Escherichia coli (Woods *et al.*, 1965) and *Aerobacter aerogenes* (Morningstar and Kisliuk, 1965) contain both types of methyltransferase. The *E. coli* non-B_{12} enzyme has a specific requirement for polyglutamate forms of CH_3-H_4PteGlu, but this may not be the case for the plant enzyme (Burton and Sakami, 1969; Dodd and Cossins, 1969). The M_r of the *E. coli* non-B_{12} enzyme is 84,000 (Whitfield *et al.*, 1970).

The *E. coli* B_{12} methyltransferase has been studied most extensively. A preparation from *E. coli* B had an M_r of 140,000 and contained 0.35 mol of B_{12} per mole protein (Taylor and Weissbach, 1973), whereas a preparation from *E. coli*

* This enzyme has also been called *methionine synthetase* (Huennekens *et al.*, 1975; Eells *et al.*, 1982). However, it is recommended (Enzyme Nomenclature Committee, 1979) that *synthetase* be applied only to enzymes catalyzing the linking together of two molecules coupled with the breaking of a pyrophosphate link in ATP. *Methionine synthase* would be an appropriate name for the methyltransferase but has been preempted by the enzyme catalyzing the synthesis of methionine from *o*-acetylhomoserine and methanethiol.

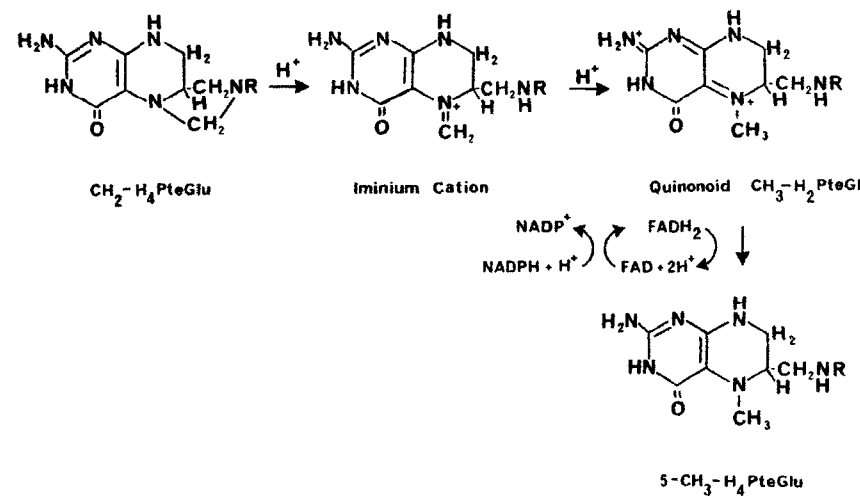

FIG. 12. Methylene tetrahydrofolate reductase reaction. R = *p*-aminobenzoyl-Glu

K12 had an M_r of 186,000 and contained 1 mol of B_{12} per mole protein (Fujii and Huennekens, 1974). Highly purified preparations of B_{12} transmethylase have been obtained from bovine kidney and brain (Mangum and North, 1971; Mangum *et al.*, 1972). Pig kidney and liver enzymes both have M_r values of 140,000 (Loughlin *et al.*, 1964; Burke *et al.*, 1971).

Vitamin B_{12} methyltransferase activation involves reduction of the B_{12} moiety to B_{12s} by a flavoprotein system followed by methylation of the cobalt by adenosylmethionine. After transfer of this methyl group to homocysteine, the cobalt is repeatedly remethylated by CH_3-H_4PteGlu. During the course of the reaction, a portion of the B_{12s} becomes oxidized and the enzyme inactivated. Activation is then brought about as described previously (Taylor and Weissbach, 1973; Huennekens *et al.*, 1975).

Vitamin B_{12} methyltransferase is important for animal metabolism because it regulates levels of H_4PteGlu via the methyl trap (Section VI,C). It also converts 5-CH_3-H_4PteGlu obtained from blood to H_4PteGlu within cells. Inhibition of the enzyme by N_2O, which binds to cobalt in the B_{12} moiety, leads to a deficiency of H_4PteGlu (Koblin *et al.*, 1982; Black and Tephly, 1983). An interesting manifestation of such a deficiency is increased sensitivity to methanol toxicity (Eells *et al.*, 1982).

Methionine represses the synthesis of the B_{12} methyltransferase in hamster cells (Kamely *et al.*, 1973). Polyglutamate forms of CH_3-H_4PteGlu are more effective than CH_3-H_4PteGlu as substrates for the B_{12} methyltransferase from rat liver (Cheng *et al.*, 1975) and bovine brain (Coward *et al.*, 1975). 5-CH_3-H_4homoPteGlu will replace 5-CH_3-H_4PteGlu as a substrate for B_{12} meth-

transferase from *E. coli* B, rabbit liver, HeLa cells, and hamster cells (Taylor and Hanna, 1974).

7, 8, and 9 *CH_2-H_4PteGlu Dehydrogenase, CH-H_4PteGlu Cyclohydrolase, and 10-CHO-H_4PteGlu Synthetase (C_1 H_4PteGlu Synthase)*

These three enzyme activities are found associated with a single multifunctional polypeptide chain in pig liver (MacKenzie, 1973; Tan and MacKenzie, 1977), sheep liver (Paukert *et al.*, 1976), rabbit liver (Schirch, 1978), chicken liver (Wasserman *et al.*, 1983), and yeast (Staben and Rabinowitz, 1983) which catalyzes the formation and interconversion of H_4PteGlu coenzymes at the formate and formaldehyde levels of oxidation (Fig. 13). The system has been called C_1 H_4PteGlu synthase, and the yeast DNA sequence corresponding to the protein has been cloned (Staben and Rabinowitz, 1983).

Treatment of the pig liver enzyme (a dimer with a subunit M_r of 100,000) with proteolytic enzymes yielded an amino terminal fragment (M_r = 33,000), which contains both dehydrogenase and cyclohydrolase activities, and a carboxy terminal fragment, which has 10-CHO-H_4PteGlu synthetase activity (Tan and MacKenzie, 1977, 1979). A channeling interaction between the dehydrogenase and cyclohydrolase has been demonstrated in which CH-H_4PteGlu, produced by the dehydrogenase reaction, reacts preferentially with the cyclohydrolase rather than equilibrating with the bulk solution (Cohen and MacKenzie, 1978; Wasserman *et al.*, 1983). The dehydrogenase and cyclohydrolase activities appear to be catalyzed at the same site (Schirch, 1978; Cohen and MacKenzie, 1980).

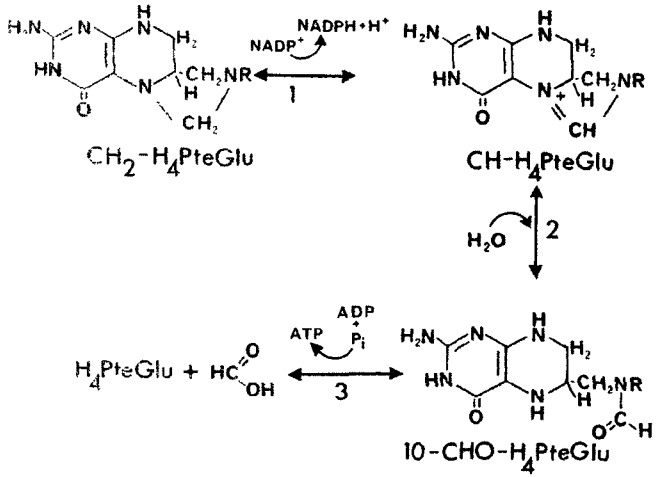

FIG. 13. Reactions catalyzed by C_1 tetrahydrofolate synthase. (1) Methylene tetrahydrofolate dehydrogenase; (2) methenyl tetrahydrofolate cyclohydrolase; (3) 10-formyl tetrahydrofolate synthetase. All three enzymes are reversible.

All three enzyme activities of C_1 H_4PteGlu synthase show much higher affinity for polyglutamate coenzymes than for monoglutamates (MacKenzie and Baugh, 1980, 1983; Wasserman *et al.*, 1983), but enhanced channeling of coenzymes was not observed with polyglutamates in the pig liver system (MacKenzie and Baugh, 1980, 1983).

The C_1 H_4PteGlu synthase copurifies with serine hydroxymethyltransferase, glycinamide ribonucleotide formyltransferase, and aminoimidazolecarboxamide ribonucleotide formyltransferase from chicken liver extracts, implying a functional association of these enzymes *in vivo* (Caperelli *et al.*, 1980; Smith *et al.*, 1980). In *Clostridium* the three activities of C_1 H_4PteGlu synthase are found in separate proteins (reviewed in Paukert *et al.*, 1976).

The 10-CHO-H_4PteGlu synthetase reaction is dependent on activation by monovalent cations. Ammonium ion is the most effective activator. The other two activities are not affected by the addition of monovalent cations (Paukert *et al.*, 1976).

10 and 11. *Glycinamide Ribonucleotide Formyltransferase and Aminoimidazolecarboxamide Ribonucleotide Formyltransferase*

These two formyltransferase reactions (Fig. 6) are on the *de novo* pathway of purine biosynthesis (Buchanan, 1982). 10-CHO-H_4PteGlu is the formyl donor for both reactions in *E. coli* (Dev and Harvey, 1978) and chicken liver (Smith *et al.*, 1981a). For many years it was believed that CH-H_4PteGlu was the formyl donor in the glycinamide ribonucleotide formyltransferase reaction because, when mixtures of disastereoisomers at C-6 of 10-CHO-H_4PteGlu or CH-H_4PteGlu were tested as substrates, only the latter mixture showed activity. It turned out, however, that the unnatural diastereoisomer of 10-CHO-H_4PteGlu is a potent inhibitor of the enzyme (K_i = 0.8 μM) so that, when the equimolar mixture of diastereoisomers is tested, no activity is seen. In contrast, with the mixture of disasteroisomers of CH-H_4PteGlu, activity is observed because (*a*) the unnatural diastereoisomer in this mixture is not an inhibitor and (*b*) contaminating cyclohydrolase activity converts the natural diastereoisomer to 10-CHO-H_4PteGlu, the correct substrate (Smith *et al.*, 1981a).

Glycinamide ribonucleotide formyltransferase and aminoimidazolecarboxamide ribonucleotide formyltransferase have been purified from chicken liver (Caperelli *et al.*, 1980); both enzymes are dimers with monomer M_r of 61,000 and 71,000, respectively. They copurify through several steps with C_1 H_4PteGlu synthase and serine hydroxymethyltransferase. The best evidence for a functional association between these enzymes was obtained with glycinamide ribonucleotide formyltransferase, which is activated by association with C_1

H_4PteGlu synthase and can be linked to it by cross-linking reagents (Smith *et al.*, 1980). Aminoimidazolecarboxamide ribonucleotide formyltransferase, however, was not stimulated by association with C_1 H_4PteGlu synthase (Mueller and Benkovic, 1981).

Analog (Smith *et al.*, 1981b) and $H_2{}^{18}O$ studies (Smith *et al.*, 1982) provide strong evidence that both formylation reactions of *de novo* purine biosynthesis *proceed by direct transfer of formyl groups without intermediate formation of* CH-H_4PteGlu or single-carbon units bound directly to the enzymes.

10-CHO-H_4PteGlu$_6$ (K_m = 1 μM) is a much more effective substrate for aminoimidazolecarboxamide ribonucleotide formyltransferase than is 10-CHO-H_4PteGlu (K_m = 674 μM) (Baggott and Krumdieck, 1979). Although unexpected, 10-CHO-H_2PteGlu$_5$ is also an excellent substrate (Baggott, 1983). The requirement for a H_4PteGlu derivative for one-carbon transfer is therefore not absolute in this instance. Methotrexate with four additional glutamate residues is a potent inhibitor of aminoimidazolecarboxamide ribonucleotide formyltransferase (K_i = 3 μM) and might play a role in the cytotoxicity of methotrexate (Baggott, 1983). Polyglutamate substrates and inhibitors of glycinamide ribonucleotide formyltransferase have not been tested as yet.

Aminoimidazolecarboxamide ribonucleotide formyltransferase and inosinicase activities reside on the same polypeptide chain that is capable of catalyzing the synthesis of inosinate from aminoimidazolecarboxamide ribonucleotide and 10-CHO-H_4PteGlu (Fig. 6) (Mueller and Benkovic, 1981).

12. *5-CHO-H_4PteGlu Cyclohydrolase (CH-H_4PteGlu Synthetase)*

$$\text{5-CHO-H}_4\text{PteGlu} + \text{ATP} \rightarrow \text{CH-H}_4\text{PteGlu} + \text{ADP} + \text{P}_i \qquad (6)$$

Highly purified proteins catalyzing this reaction have been prepared. Preliminary studies of the homogeneous rabbit liver enzyme show it to have an M_r of 25,000 (Hopkins and Schirch, 1983). The pure *L. casei* enzyme (M_r = 23,000) is not inhibited by 500 μM methotrexate, 5-CH_3-H_4PteGlu, or folate (Grimshaw *et al.*, 1983).

It is likely that this enzyme is responsible for catalyzing the conversion of 5-CHO-H_4PteGlu to CH-H_4PteGlu *in vivo* and is therefore worthy of consideration as a modulating influence on the rescue of cells from methotrexate toxicity by 5-CHO-H_4PteGlu.

13 and 14. *Formiminoglutamate:Tetrahydrofolate Formiminotransferase and Formiminotetrahydrofolate Cyclodeaminase*

These enzyme activities (Fig. 14) are on the pathway of histidine catabolism in animals. Folate deficiency can lead to the excretion of formiminoglutamate in the urine (Luhby and Cooperman, 1964). As isolated from pig liver, formiminotransferase–cyclodeaminase is a tetramer of dimers (MacKenzie *et al.*, 1980).

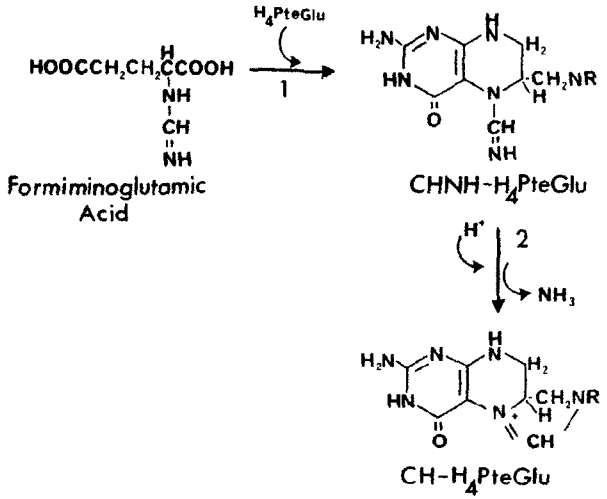

FIG. 14. Reactions catalyzed by (1) formiminoglutamate:tetrahydrofolate formiminotransferase and (2) formiminotetrahydrofolate cyclodeaminase.

Each monomer (M_r = 62,000) is a bifunctional polypeptide that catalyzes both reactions.

Substrate channeling is observed only when polyglutamate substrates are used; that is, CHNH-H_4PteGlu$_5$ transfers the CHNH-H_4PteGlu moiety to the deaminase site before equilibrating with the medium (MacKenzie and Baugh, 1980). The K_m values are 48 μM for H_4PteGlu and 3.5 μM for H_4PteGlu$_5$.

Formiminoglutamate formiminotransferase also catalyzes the transfer of the 5-CHO group of 5-CHO-H_4PteGlu to glutamate, forming *N*-formyl-Glu plus H_4PteGlu (Silverman *et al.*, 1957; Tabor and Wyngarden, 1959; Bortoluzzi and MacKenzie, 1983). All of the formyltransferase activity in liver extracts can be accounted for by the formiminotransferase present. The V_{max} of the formiminotransferase with 5-CHO-H_4PteGlu as substrate is 0.03% that obtained with CHNH-H_4PteGlu as substrate.

The unnatural diastereoisomer of H_4PteGlu inhibits the deaminase reaction (MacKenzie and Baugh, 1983).

15. *10-CHO-H_4PteGlu Dehydrogenase*

This enzyme, prepared in homogeneous form from rat liver, is a tetramer (monomer M_r = 108,000; Scrutton and Beis, 1979) that catalyzes two reactions:

$$\text{10-CHO-H}_4\text{PteGlu} + \text{NADP}^+ + \text{H}_2\text{O} \rightarrow \text{H}_4\text{PteGlu} + \text{CO}_2 + \text{NADPH} + \text{H}^+ \qquad (7)$$

$$\text{10-CHO-H}_4\text{PteGlu} + \text{H}_2\text{O} \rightarrow \text{H}_4\text{PteGlu} + \text{HCOOH} \qquad (8)$$

A partially purified preparation from pig liver catalyzes the same two reactions (Kutzbach and Stokstad, 1971b). The V_{max} of Eq. (8) ($NADP^+$ omitted) is 20% that of Eq. (7). In the presence of $NADP^+$ Eq. (8) is not observed.

H_4PteGlu is a potent product inhibitor of the enzyme. The K_i for the natural diastereoisomer is 1 μM. It is reasonable to suppose that polyglutamate forms of H_4PteGlu would be even more inhibitory. Thus, the presence of free H_4PteGlu polyglutamates would lead to the conservation of single-carbon units by preventing their oxidation to CO_2. Equation (7) is irreversible (Kutzbach and Stokstad, 1971b).

5-CH_3-H_4PteGlu, 5-CHO-H_4PteGlu, PteGlu, aminopterin, and H_4-aminopterin do not inhibit the pig liver enzyme at 500 μM (Kutzbach and Stokstad, 1971b). Product inhibition of Eq. (7) by NADPH could not be demonstrated (Scrutton and Beis, 1979). This substance serves rather as an activator of the reaction. Enzyme activity of the rat liver preparation was not altered by methionine or adenosylmethionine (Scrutton and Beis, 1979). Formate oxidation to CO_2 is greatly diminished in folate-deficient rats (Friedman *et al.*, 1954). The combined action of 10-CHO-H_4PteGlu synthetase and 10-CHO-H_4PteGlu dehydrogenase is a likely pathway for the folate-dependent formate oxidation.

16 and 17. Dimethylglycine Dehydrogenase and Sarcosine Dehydrogenase

Two flavoproteins in rat liver mitochondria are capable of binding folates. These are dimethylglycine dehydrogenase (M_r = 90,000) and sarcosine dehydrogenase (M_r = 105,000) (Fig. 15) (Wittwer and Wagner, 1981). Dimethylglycine dehydrogenase catalyzes the oxidation of the methyl group of sarcosine in addition to those of dimethylglycine, but sarcosine dehydrogenase is more specific in its action and does not catalyze the oxidation of the methyl groups of dimethylglycine.

The relevant literature in this area has been reviewed by Wittwer and Wagner (1981). It is suggested that dimethylglycine dehydrogenase interacts with dimethylglycine and sarcosine arising from choline, whereas sarcosine dehydrogenase utilizes sarcosine arising from the methylation of glycine by adenosylmethionine. The latter reaction is postulated to play a role in the regulation of adenosylmethionine levels in the cytosol (Kerr, 1972). The transport of this

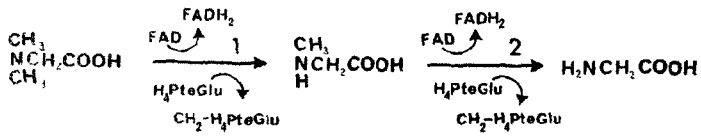

FIG. 15 Reactions catalyzed by (1) dimethylglycine dehydrogenase and (2) sarcosine dehydrogenase.

sarcosine into the mitochondria and its subsequent oxidative conversion to serine may serve as a mechanism for moving one-carbon units into mitochondria. It is likely that sarcosine dehydrogenase and serine hydroxymethyltransferase are closely juxtaposed in mitochondria because glycine produced from sarcosine is used in preference to exogenous glycine for serine formation (Mackenzie, 1955).

Both dehydrogenases produce formaldehyde from their respective substrates in the absence of H_4PteGlu (Wittwer and Wagner, 1981). As isolated, however, both dehydrogenases contain 1 mol of folate, which is in the form of either H_4PteGlu or H_4PteGlu$_5$. The respective dissociation constants for H_4PteGlu and H_4PteGlu$_5$ for dimethylglycine dehydrogenase are 0.4 and 0.2 μM. Thus, both the monoglutamate and the pentaglutamate have very high affinity for this enzyme.

B. Folate Enzymes of Specialized Function

With the exception of methionyl-tRNA formyltransferase, which is found in animal mitochondria, all of the folate enzymes listed in Table IX are from bacteria.

Ribothymidyl synthase is an interesting variation of thymidylate synthase. Ribothymidine usually arises by methylation of a uracil residue in tRNA by adenosylmethionine. However, in *Streptococcus faecalis* (Delk *et al.*, 1980) ribothymidine results from a reaction of tRNA with CH_2-H_4PteGlu in which the reducing equivalents used to form the methyl group arise from $FADH_2$ rather than H_4PteGlu, as is the case with thymidylate synthase. Neither adenosylmethionine nor 5-CH_3-H_4PteGlu is an intermediate in the ribothymidyl synthase reaction. This system is also found in *Bacillus subtilis* (Delk *et al.*, 1976).

VIII. Folate Polyglutamates

A. Introduction

An understanding of the metabolic role of polyglutamate derivatives of folates and folate analogs is essential to the interpretation of data on folate function as well as the chemotherapeutic action of antifolates. We summarize here the more recent literature. Further information in this area can be found in the following: Baugh and Krumdieck (1971), Covey (1980), Cichowicz *et al.* (1981), McGuire and Bertino (1981), Kisliuk (1981), Goldman *et al.* (1983), and Cheng (1983).

B. Distribution

The polyglutamate chain lengths found in the total folate pool of several organisms and tissues are given in Table XV. The vast majority of folates are present as polyglutamate forms. In some organisms a single polyglutamate chain length predominates, whereas others contain a broad distribution. Coliphage

TABLE XV

DISTRIBUTION OF FOLYL POLYGLUTAMATE CHAIN LENGTHS IN VARIOUS ORGANISMS AND TISSUES[a]

Source	Number of glutamate residues									Reference
	1	2	3	4	5	6	7	8	9	
Prokaryotes										
Clostridium acidi-urici			100							Curthoys and Rabinowitz (1972)
Corynebacterium				100						Foo *et al.* (1980)
Escherichia coli B	11		48	16	12	8	4	1		Kozloff *et al.* (1979a)
Coliphage T4D						100				Kozloff *et al.* (1979a)
Lactobacillus casei										
High-PteGlu medium	3		9	59	23	5				Baugh *et al.* (1974)
Low-PteGlu medium						8	14	42	19	Scott (1976) Shane *et al.* (1983)
Eukaryotes										
Monkey liver				24	46	15				Brown *et al.* (1974)
Hamster liver				30	51	13	2			Scott (1976)
Rat liver										
Normal				2	53	42	2			Eto and Krumdieck (1982)
Regenerating				1	20	59	20			Eto and Krumdieck (1982)
Saccharomyces cerevisiae						12	71	13		Scott (1976)
Neurospora crassa										
Regular medium					14	80		6^b		Chan and Cossins (1980)
High-glycine medium						56	24	20^b		Chan and Cossins (1980)

[a]Numbers represent percentage of total PteGlu.
[b]Eight or more glutamate residues.

T4D contains $H_2PteGlu_6$ as a structural component of its base plate. Neither $H_2PteGlu_5$ nor $H_2PteGlu_7$ will serve in its place. Most of the polyglutamate chains found in the host are shorter. However, T4D infection of *E. coli* causes the formation of very long chains, which are cleaved to $H_2PteGlu_6$ by T4D-induced γ-glutamate hydrolase activity (Kozloff *et al.*, 1979b, 1983; Kozloff, 1980).

Polyglutamate chain length may change under different dietary or growth conditions (Table XV). There is a shift to longer glutamate chains in *Neurospora* grown on a high-glycine medium, in *Lactobacillus casei* grown on a low-PteGlu medium, in quail liver from animals maintained on a high-casein diet, and in rats given a folate-deficient diet (Cassady *et al.*, 1980). In cultured mouse hepatoma

cells starved of folate for 48 h, Glu_8 is the predominant folate polyglutamate as opposed to the normal distribution of equal amounts of Glu_5 and Glu_6 (Priest *et al.*, 1983). The reason for these shifts is not known, but all of these situations might benefit if increased efficiency of PteGlu coenzyme activity is brought about by longer glutamate chains.

Until relatively recently, technical difficulty prevented examination of the changes in polyglutamate chain length associated with individual PteGlu forms such as CH_3-H_4PteGlu or CHO-H_4PteGlu. Eto and Krumdieck (1982) have reported that, during rat liver regeneration, Glu_6 and Glu_7 derivatives increase at the expense of Glu_4 and Glu_5 forms. The newly synthesized Glu_6 forms are predominantly CH_3-H_4PteGlu, whereas the newly synthesized Glu_7 forms are formylated derivatives. Brody *et al.* (1982) found that different H_4PteGlu derivatives predominate in fractions of rat liver separated according to glutamate chain length. Among Glu_5 derivatives, CH_3-H_4PteGlu is the predominant form, whereas the Glu_7 forms are mainly H_4PteGlu. These results imply that PteGlu derivatives containing different glutamate chain lengths may serve different functions and may be located in different cellular compartments.

Other biological materials found to contain folate polyglutamates include *Physalia physalis* (Portuguese man-of-war) (Wittenberg *et al.*, 1962), soybeans (Iwai and Nakagawa, 1958), wheat, pea, spinach, radish, and corn plants (Clandinin and Cossins, 1972; Spronk and Cossins, 1972), *Euglena gracilis* (Lor and Cossins, 1973), plant mitochondria and chloroplasts (Cossins and Shah, 1972), and human red blood cells (Benesch *et al.*, 1983). In *Physalia* the folate polyglutamates are likely to be involved in the formation of CO (found in the float gas) from the β-carbon of serine.

C. BIOSYNTHESIS

Radiolabeled folate is readily incorporated into polyglutamate forms in animals. Evidence so far indicates that animal tissue uses a single enzyme to add all of the glutamate residues (McGuire *et al.*, 1980; Foo and Shane, 1982). In *Neurospora*, however, genetic and enzyme studies show that the enzyme forming Glu_2 derivatives differs from the enzyme that adds the remaining residues (Cossins and Chan, 1983).

$PteGlu_n$ synthetases have been examined in *Escherichia coli* (Ferone and Warskow, 1983; Ferone *et al.*, 1983), *Corynebacterium* (Shane, 1980), *L. casei* (Bognar and Shane, 1983), hamster cells (Taylor and Hanna, 1977), sheep liver (Gawthorne and Smith, 1973), rat liver (McGuire *et al.*, 1980; Priest *et al.*, 1981b), and pig liver (Cichowicz *et al.*, 1981). All of these systems require Mg^{2+} and K^+ for activity and have high pH optima, ranging from 8.4 in the sheep and rat systems to 10 in *Corynebacterium*. Adenosine triphosphate is required with dATP having equal or greater activity. Specificity for glutamate incorporation is observed; neither aspartate, glycine, methionine, nor a wide variety of potential glutamate replacements is active. H_4PteGlu or CH_2-

H_4PteGlu is generally the most active substrate, with 5-CH_3-H_4PteGlu being considerably less active. A pteroate moiety is required for activity, and many *conjugates of PteGlu including aminopterin, methotrexate, homofolate,* and the unnatural diastereoisomer of H_4PteGlu are substrates. The synthesis of polyglutamate derivatives of these analogs must be considered in evaluating their cytotoxicity in view of the inhibitory potency of polyglutamate analogs for many enzyme systems (Kisliuk, 1981; Baggott, 1983; Table XIV).

Product inhibition (McGuire *et al.*, 1980) and substrate specificity (Foo and Shane, 1982) have been suggested to be mechanisms by which the chain length found *in vivo* can be determined. An enzyme preparation from rat liver was found to catalyze the exchange of the terminal γ-glutamate residue with methionine, glutamate, glutamine, or glycine (Brody and Stokstad, 1982). The physiological significance of this activity is not known.

D. Degradation

γ-Glutamate hydrolases are often called *conjugases* because they can form $PteGlu_1$ and $PteGlu_2$ derivatives from conjugated ($PteGlu_n$) forms. The γ-glutamate hydrolases that have been highly purified are listed in Table XVI along with some of their properties. Enzymes from different sources cleave the γ-glutamate chain at different points, but all require a free carboxy terminal residue for activity. One of the most widely used preparations is from hog kidney, but it has not been highly purified. It liberates $PteGlu_1$ forms.

The γ-glutamate hydrolases having a pH optimum near 4.5 are considered to be of lysosomal origin. A comparison of $PteGlu_n$ hydrolases in mouse tissues (Priest *et al.*, 1982) showed that kidney and muscle extracts generate intermediates of all chain lengths from $PteGlu_5$ on route to PteGlu, whereas liver extracts yield PteGlu directly. Similar results were obtained with isolated lysosomes from the respective tissues. It is not certain if these enzymes are related to folate metabolism *in vivo*.

The pancreatic and intestinal enzymes with higher pH optima are most likely involved in the conversion of dietary $PteGlu_n$ derivatives to $PteGlu_1$ and $PteGlu_2$ derivatives to facilitate intestinal absorption. $PteGlu_n$ hydrolysis occurs in the intestinal mucosa as part of the intestinal absorption process (Butterworth *et al.*, 1969). Long-chain PteGlu derivatives are not absorbed by the intestine (Baugh *et al.*, 1975; for a review see Halsted, 1979).

Animal γ-glutamate hydrolases cannot cleave the α-amide bond between the pteroate and glutamate residues. Certain bacterial carboxypeptidases catalyze this reaction (Pratt *et al.*, 1968; Levy and Goldman, 1968; Albrecht *et al.*, 1978).

E. Coenzyme Function

Polyglutamyl derivatives of folate are generally regarded as the active coenzyme forms in tissues because (*a*) folates are universally present in tissues as

TABLE XVI

Properties of Some Poly-γ-glutamate Hydrolases

Source	Action	M_r	pH optimum	References
Beef liver[a]	Endopeptidase $PteGlu_n \rightarrow PteGlu_1 + Glu_{n-1}$ $Glu_{n-1} \rightarrow (n-1)(Glu_1)$	108,000	4.5	Silink *et al.* (1975)
Chicken intestine	Endopeptidase $PteGlu_n \rightarrow PteGlu_2 + Glu_{n-2}$	80,000	7.5	Rosenberg and Neumann (1974)
Chicken liver	Exopeptidase (carboxypeptidase) $PteGlu_n \rightarrow PteGlu_1 + (n-1)(Glu_1)$	60,000	4.1, 5.2	Rao and Noronha (1977a,b)
Chicken pancreas	Exopeptidase (carboxypeptidase) $PteGlu_n \rightarrow PteGlu_2 + (n-2)(Glu_1)$	130,000	7.8	Kaferstein and Jaenicke (1972)

[a]The beef liver enzyme is a glycoprotein containing Zn^{2+}.

polyglutamates. (*b*) polyglutamate cofactors generally have a higher affinity for folate enzymes than the corresponding monoglutamates (see McGuire and Bertino, 1981; Cichowicz *et al.*, 1981; Kisliuk, 1981, for listings of K_m values), and (*c*) a mutant Chinese hamster ovary cell line that lacks the enzyme folylpolyglutamate synthetase but is normal in all other respects has growth requirements for glycine, adenine, and thymidine (Taylor and Hanna, 1977). These mutant cells cannot carry out normal biosyntheses when lacking folate polyglutamates. In a medium lacking only thymidine the mutant cells grow on high concentrations of 5-CHO-H_4PteGlu but, if either glycine or adenine is lacking, 5-CHO-H_4PteGlu will not support growth. It appears that the enzymes required for thymidylate biosynthesis, namely, serine hydroxymethyltransferase, thymidylate synthase, and dihydrofolate reductase, are less sensitive to a lack of polyglutamate coenzyme than the enzyme systems required for glycine or adenine synthesis.

Evidence is accumulating that the polyglutamate chain serves a coordinating function in one-carbon metabolic pathways. It seems reasonable to suppose that the carboxy terminal portion of the polyglutumate chain is attached to an anchor protein, perhaps distinct from any known folate enzyme. The PteGlu portion of the molecule then proceeds sequentially from enzyme to enzyme (MacKenzie and Baugh, 1980; Kisliuk and Gaumont, 1983).

F. Regulatory Function

Cystathionine γ-synthase from *Neurospora crassa* catalyzes the formation of cystathionine from *O*-acetylhomoserine and cysteine. The cystathionine is cleaved to homocysteine, which yields methionine after reacting with 5-CH_3-H_4PteGlu. Cystathionine γ-synthase has an absolute requirement for 5-CH_3-H_4PteGlu as an allosteric activator whereby the activator stimulates the formation of the acceptor of its methyl group. 5-CH_3-H_4PteGlu$_7$ is much more active than 5-CH_3-H_4PteGlu as an activator. *S*-Adenosylmethionine is an allosteric inhibitor of cystathionine γ-synthase. Thus, CH_3-H_4PteGlu$_7$ and *S*-adenosylmethionine reciprocally control the synthesis of the methyl group acceptor homocysteine (Selhub *et al.*, 1971).

Serine hydroxymethyltransferase is also regulated by CH_3-H_4PteGlu. Its weak inhibition (Schirch and Ropp, 1967) is enhanced by elongation of the glutamate chain (Matthews *et al.*, 1982). Inhibition of serine hydroxymethyltransferase leads to a decrease in the amount of serine used to supply single-carbon units for purine, pyrimidine, and methionine formation and an increase in the amount of serine used for gluconeogenesis and/or oxidative removal.

A role for H_2PteGlu$_n$ in regulating the activities of thymidylate synthase (Kisliuk *et al.*, 1974) and CH_2-H_4PteGlu reductase (Matthews and Baugh, 1980) has been suggested. Each of these enzymes is strongly inhibited by H_2PteGlu$_n$, and each has CH_2-H_4PteGlu$_n$ as its substrate. A decrease in the CH_2-H_4PteGlu$_n$/H_2PteGlu$_n$ ratio would result in decreased CH_2-H_4PteGlu$_n$ reduction to form methyl groups, shunting the CH_2 group to oxidative pathways and preventing the further formation of H_2PteGlu by the action of thymidylate synthase. H_2PteGlu$_5$ enhances the binding of fluorodeoxyuridylate to thymidylate synthase, which could explain the cytotoxic synergy of fluorouracil and methotrexate in L1210 cells (Fernandes and Bertino, 1980).

IX. Addendum

Areas of folate metabolism not covered in this chapter but with relevance to chemotherapy with antifolates include (*a*) folate-binding proteins that could be involved in folate transport and storage (Colman and Herbert, 1980; Suzuki and Wagner, 1980; Colman *et al.*, 1981), (*b*) intestinal absorption of folates (Halsted, 1979), (*c*) folate distribution in tissues (Scott *et al.*, 1983), (*d*) the effects of alcohol (Hillman and Steinberg, 1982) and contraceptive steroids (Lakshmaiah and Bamji, 1981) on folate absorption and distribution, (*e*) folate nutrition (Broquist *et al.*, 1977, and (*f*) folates in neurology (Botez and Reynolds, 1979).

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CHAPTER TWO

The Comparative Biochemistry of Dihydrofolate Reductase

James H. Freisheim

Department of Biological Chemistry
University of Cincinnati College of Medicine
Cincinnati, Ohio

David A. Matthews

Department of Chemistry
University of California at San Diego
La Jolla, California

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A PHASE I AND PHARMACOKINETIC (PK) STUDY OF THE MULTITARGETED ANTIFOL (MTA) LY231514 WITH FOLIC ACID (Meeting abstract).

Sub-category: Other
Category: Clinical Pharmacology
Meeting: 1998 ASCO Annual Meeting

Abstract No: 866

Author(s): L Hammond, M Villalona-Calero, SG Eckhardt, R Drengler, C Aylesworth, T Johnson, M Hidalgo, G Rodriguez, S Diab, P Monroe, D Thornton, Hoff D Vo, E Rowinsky

Abstract: MTA (LY 231514) is a new antifol that inhibits multiple folate-dependent enzymes, including thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyl transferase. Initial phase I trials demonstrated major antitumor responses when MTA was given as a 10 min I.V. infusion, however, myelosuppression precluded dose escalation above 500-600 mg/m2. Since preclinical studies indicated that folic acid supplementation increases the eherapeutic index of MTA, the feasibility of administering folic acid 5 mg daily for 5 days starting 2 days before MTA in minimally- and heavily-pretreated pts was evaluated to determine if folic acid supplementation ameliorates the toxif effects of MTA, permitting significant dose-escalation above the recommended phase II dose of MTA alone. Thus far, 21 pts with solid cancers have received 55 courses at the following dose levels: 600, 700, and 800 mg/m2. Drug-related toxicities have included neutropenia, anemia, and thrombocytopenia, which have been more severe in heavily-pretreated pts. Other toxicities (grade 1-2) include rash, somnolence, fatigue, leg edema, and diminished renal function manifested by a decrease in creatinine clearance. One pt taking a non-steroidal anti-inflammatory agent experienced severe toxicities at the 800 mg/m2 dose, which resolved after administration of leucovorin and thymidine. One partial response in a pt with metastatic colon cancer has been observed. PK and vitamin (folic acid) metabolite profiles were done during cycles 1 and 3 at 600 to 800 mg/m2. To date, serum folic acid levels do not appear to be related to toxicity, but homocysteine was significantly elevated in the pt with severe toxicities at the 800 mg/m2 dose. Thus far, heavily- and minimally-pretreated patients have tolerated MTA at 600 and 800 mg/m2 and accrual continues at 700 and 900 mg/m2, respectively. These results indicate that folic acid supplementation appears to permit MTA dose escalation.

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TABLE 29-16. Toxicity Symptoms Reported to Be Associated with Chronic High Doses of Pyridoxine

Symptoms
Motor and sensory neuropathy; vesicular dermatosis on regions of the skin exposed to sunshine.
Peripheral neuropathy; loss of limb reflexes; impaired touch sensation in limbs; unsteady gait, impaired or absent tendon reflexes; sensation of tingling that proceeds down neck and legs
Dizziness; nausea; breast discomfort or tenderness
Photosensitivity on exposure to sun

Source: Leklem LE. Vitamin B6. In: Machlin LJ, ed. Handbook of vitamins. 2nd ed. New York: Marcel Dekker, 1991.

conditions of vitamin B_6 deficiency, the enzyme is not saturated by coenzyme *in vivo*, and the activity ratio will exceed 1.5 and 1.25, respectively.[223] An elevated erythrocyte AST (EAST) index or ratio is a commonly accepted indicator of inadequate B_6 nutriture.

An older procedure for determination of B_6 nutritional status is the tryptophan loading test. Urine is collected for 24 hours after ingestion of 2 to 5 g of L-tryptophan, and output of xanthurenic acid is measured. In vitamin B_6 deficiency, kynureninase activity is decreased, and kynurenine and 3-hydroxykynurenine accumulate. There is a resultant increase in excretion of tryptophan metabolites, including xanthurenic acid (see Fig. 29-20). A similar protocol is employed in the methionine loading test, with assessment of cystathionine excreted being used to evaluate B_6 status. Other widely used methods for vitamin assessment have included microbiologic and fluorometric assays.[225]

The concentration of plasma PLP is considered to be the best indicator of vitamin B_6 status, including tissue stores. The 4-pyridoxic acid content of a 24-hour urine reflects the production and excretion of the major metabolite of B_6. Reduced excretion of this urinary metabolite is one of the earliest indicators of a B_6 deficiency.

Direct assessment of B_6 levels is complicated by photosensitivity of the vitamers. HPLC methods for measurement of 4-pyridoxic acid levels in the urine or B_6 vitamers in the plasma are rapid, specific, and sufficiently sensitive to be clinically useful.[8,65,218] A sensitive and reliable procedure for determination of PLP by HPLC with electrochemical detection has been described.[47]

Also of interest is a radioenzymatic assay for direct measurement of PLP, based on activity of the PLP-dependent enzyme tyrosine decarboxylase from *Streptococcus fecalis*.[34] The commercially available apoenzyme is incubated with tritiated tyrosine and patient plasma. PLP in the specimen provides the required coenzyme, and the decarboxylated metabolite formed ([^{3}H]tyramine) is extracted and quantified by liquid scintillation counting.

Folates

Folates comprise a family of compounds derived from folic or pteroylglutamic acid.[192] All members of the family possess the double-ring structure pteridine (2-amino-4-hydroxy-6-methylpterin) joined by a methylene bridge to *para*-aminobenzoic acid (PABA). This parent compound is called *pteroic acid (Pte)*. PABA, in turn, is linked through a peptide bond to one molecule of glutamic acid, forming folic acid (FA) or pteroylglutamic acid (PteGlu; PGA) (Fig. 29-22). Conjugation with additional glutamic acid residues produces a series of polyglutamates. The bulk of the vitamin is present in the diet as folate polyglutamates. Enzymes requiring folic acid as a coenzyme catalyze chemical reactions involving the transfer and utilization of single carbon units. Nitrogen atoms at the 5 and 10 positions in the pteridine ring portion of the molecule are active in these single carbon unit transfers. The polyglutamate chain attaches the coenzyme to the apoenzyme. Double bond reduction and presence of various substituents serve to differentiate the various analogs of folic acid. Reduction of double bonds between ring positions 5–6 and 7–8 converts folic acid into tetrahydrofolic acid (THFA, or FH_4). The term *folate* is applied generically to the entire group of compounds. Use of the older generic descriptor, folacin, is no longer acceptable.

The most recently published folate RDAs are 180 μg for adult females and 200 μg for adult males.[181] For adolescents 150 μg is recommended. The minimal daily requirement for folate is approximately 50 μg for adults.[100] Use of oral contraceptive steroids can increase urinary excretion of folate. Increased vitamin intake may be required to offset the loss.[212,229] In pregnancy, the RDA is raised to 400 μg to maintain maternal folate reserves and adequately support normal fetal growth (see Table 29-4).[181] Megaloblastic anemia of pregnancy is commonly due to folate deficiency. Folic acid, even as much as 15 mg daily over several years, is reportedly not toxic in humans.[103] However, some data suggest that excessive intake of supplemental folate may interfere with intestinal absorption of zinc.[31,170]

The name *folate*, like the word foliage, is derived from the Latin word for leaf. Cruciferous vegetables, such as spinach, turnip greens, asparagus, broccoli, and brussels sprouts, are

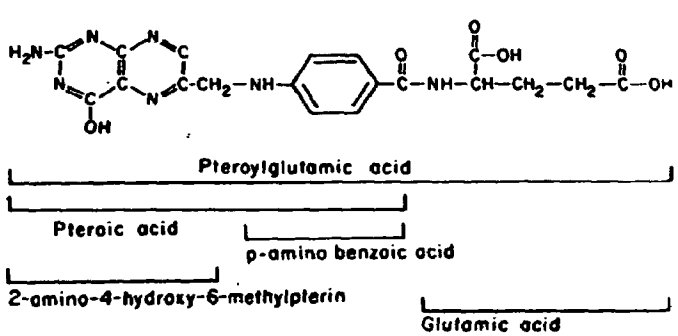
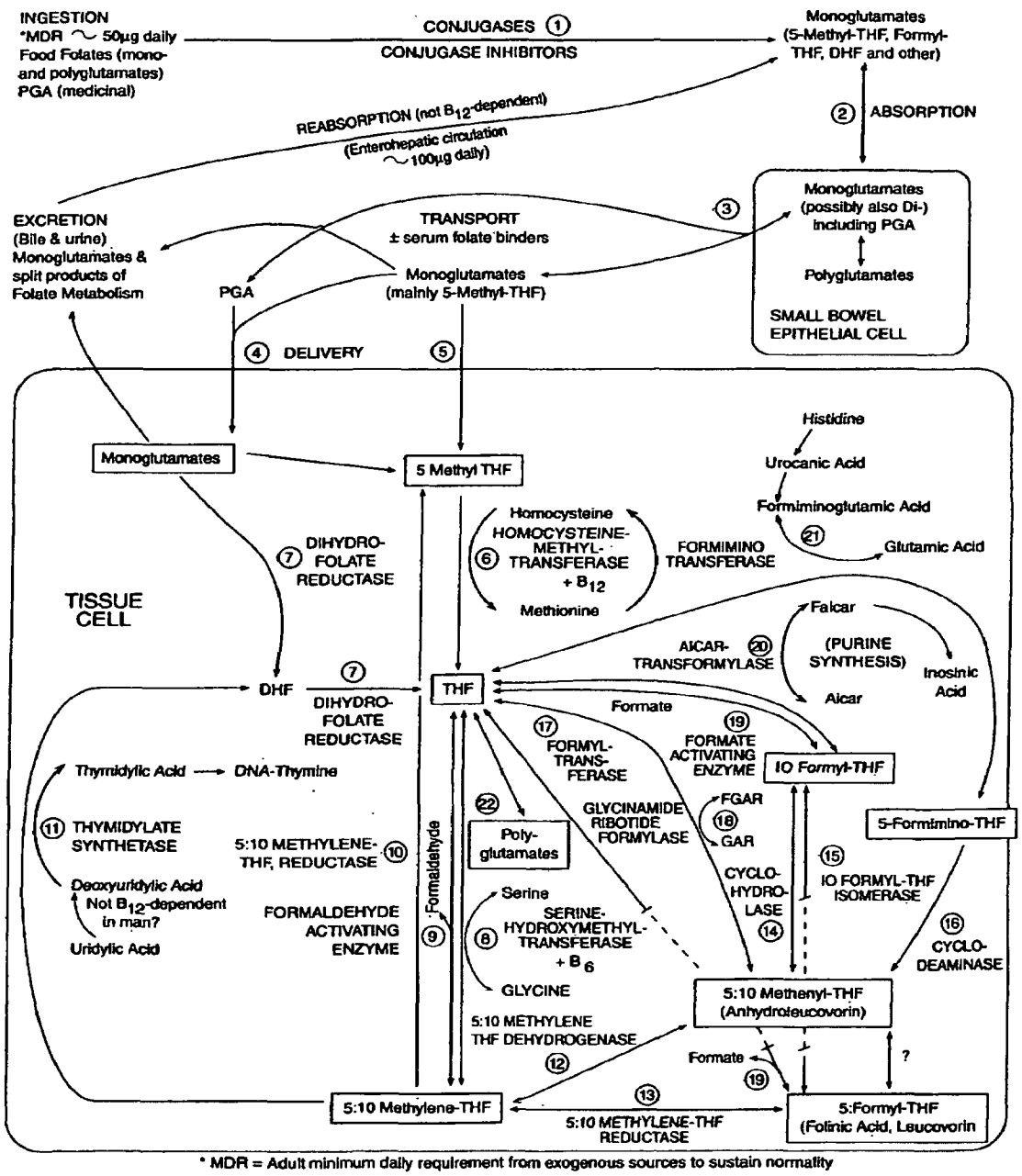


Figure 29-22. Folacin.

rich in folate. Folate is abundant in liver, kidney, whole-grain cereals, yeast, and mushrooms.[82] The vitamin is also synthesized by intestinal microflora. Prolonged cooking, particularly steaming and boiling, destroys most folate in foods. Infants receiving boiled formulas prepared with pasteurized, sterilized, or powdered cow's milk require folate supplementation.

Following ingestion, polyglutamates are enzymatically hydrolyzed to monoglutamates by action of conjugases in the mucosa of the small intestine (Fig. 29-23). Folate monoglutamates are rapidly absorbed and transported in the circulation mainly as the tetrahydrofolate (FH_4) derivative. The major form of folate in serum and red cells is 5-methyl-tetrahydrofolate (N^5-methyl-FH_4). Dihydrofolate reductase



▲ *Figure 29-23.* Flow chart of folate metabolism in humans. Circled numbers indicate individual steps in folate metabolism. *Source:* Herbert V, Das KC. Folic acid and vitamin B12. In: Shils ME, Olson JA, Shike M, eds. Nutrition in health and disease. 8th ed. Vol.1. Philadelphia, PA: Lea & Febiger, 1994.

catalyzes the enzymatic reduction reaction.[42] Folate may circulate in the free form or attached to low-affinity or high-affinity binders. Approximately two-thirds of folate is loosely bound to plasma proteins, including albumin, α_2-macroglobulin, and perhaps, transferrin. High-affinity folate-binding proteins have been purified from serum, milk, and cerebrospinal fluid. The role these specific proteins play in overall folate nutriture is not clear. The milk protein could facilitate intestinal uptake of folate. Presence of a folate-binding protein in the choroid plexus may account for the high CSF/serum ratio of the vitamin. Serum folate levels range from 3 to 25 ng/mL.[42] Marginal deficiency is suggested by concentrations from 3 to 5 ng/mL; levels above 5 ng/mL are interpreted as indicating adequate folate.[28,100] Recent efforts to establish a pediatric reference range show folic acid concentrations to be higher in children, especially in those less than 1 year of age, than in adults. During adolescence, a significant decrease in serum folic acid concentration has been noted.[102] Folate concentration in CSF ranges from 15 to 35 ng/mL.[42] While folate monoglutamates are the circulating and transport forms, polyglutamates are the primary intracellular storage forms of the vitamin.[100] Hepatic stores are believed to account for approximately 50% of the body's reserve of folate, predominantly as pentaglutamates. Other tissues with high concentrations of folate are the kidney and blood cells. RBC folate is almost entirely in the form of methylfolate pentaglutamates. Negative folate balance is indicated by erythrocyte vitamin levels less than 200 ng/mL; tissue depletion occurs when folate levels fall below 160 ng/mL.[100] Tissue folate-binding proteins are reported in granulocytes as well as the brush border of intestinal mucosa. Leukocyte folate ranges from 60 to 123 μg/L of WBCs.[42] Folate-requiring enzymes serve as intracellular folate binders. Excretion occurs in the bile and urine (Fig. 29-23).

Vitamin deficiency may be dietary in origin, associated with malabsorption, or drug-induced (Table 29-17). Nutritional folate deficiency is seen in infants raised on goat's milk, which has only about 10% of the concentration of the vitamin found in human or cow's milk.[181] Inborn errors of folate metabolism (*e.g.*, dihydrofolate reductase deficiency and congenital folate malabsorption) give rise to folate deficiency. Total parenteral nutrition using amino acid solutions, unsupplemented by folate, has been reported to induce acute depression of serum folate, marked by pancytopenia and megaloblastic anemia.[73] Folate malabsorption may occur in conditions such as Crohn's disease or ulcerative colitis. Ironically, sulfasalazine, which is used in the treatment of inflammatory bowel disease, impairs folate absorption. Vitamin deficiency may arise during anticonvulsant therapy with phenytoin or phenobarbital.[31] Other drugs that affect folate status include cycloserine, metformin, and cholestyramine. Antifolate medications are used in the treatment of a wide range of malignant and nonmalignant disorders.[100] Folate antagonists appear to bind irreversibly to the enzyme dihyrofolate reductase. Examples of such drugs are triamterene, a diuretic; pyrimethamine, an antimalarial; trimethoprim, an antimalarial as well as a potentiator of sulfonamides in the

TABLE 29-17. Diseases Treated with Drugs Known to Interfere with Folate Metabolism

Disease	Drug
Cancer, leukemia	Methotrexate
Psoriasis	Methotrexate
Rheumatoid arthritis	Methotrexate
Bronchial asthma	Methotrexate
Bacterial infection	Trimethoprim
Malaria	Pyrimethamine
Hypertension	Triamterene
Crohn's disease	Sulfasalazine
Gout	Colchicine
Epilepsy	Phenytoin
AIDS	Trimetrexate

Source: From Butterworth CE, Tamura T. Folic acid safety and toxicity: A brief review. Am J Clin Nutr 1989;50:353.

treatment of bacterial infections; and pentamidine, used in treatment of trypanosomiasis and leishmaniasis.[212] Pentamidine is also employed in the treatment of pneumonia, presumably due to protozoal infection. Pulmonary disease caused by *Pneumocystis carinii* occurs in 65% to 85% of all AIDS patients. The most common manifestation of this infection is pneumonia. Among the adverse reactions arising from standard pentamidine therapy in the treatment of this pneumonia is the development of folate deficiency. The cancer chemotherapeutic agent methotrexate (MTX) is an especially potent folate antagonist.[212] MTX may also be utilized in the treatment of psoriasis and rheumatoid arthritis. The acute toxicity of folate antagonists is due to their impairment of DNA synthesis. A pharmacologic amount (>0.4 mg/d) of folic acid may be administered as a "rescue dose" to patients receiving cancer chemotherapy.

In the U.S., inadequate folate nutriture is particularly common among those in lower socioeconomic groups.[28] Folic acid deficiency has been reported as the most common nutritional deficiency among low-income and institutionalized elderly.[28,167,216] Exposure to ethanol may alter the activity of intestinal brush border folate hydrolase (conjugase), causing malabsorption of the vitamin. Alcohol also interferes with hepatic processing of folate. Chronic alcoholism is a major cause of folate deficiency in the United States.

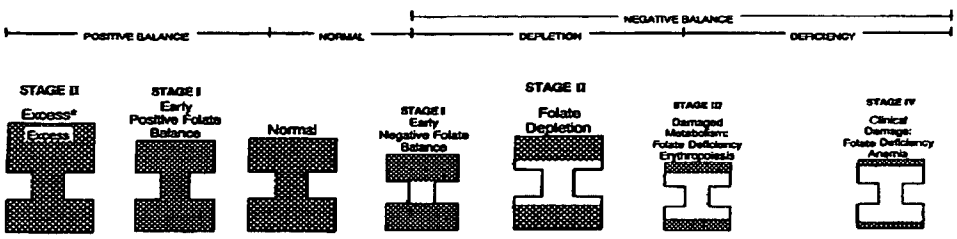
Tetrahydrofolate (THF) derivatives serve as cofactors for enzymes catalyzing the transfer of 1-carbon groups in methylation reactions necessary for a variety of biochemical reactions. The coenzyme forms of the vitamin include the following tetrahydrofolates: N^5-formyl-FH_4; N^{10}-formyl-FH_4; N^5-formimino-FH_4; N^5, N^{10}-methenyl-FH_4; N^5, N^{10}-methylene-FH_4; and N^5-methyl-FH_4.[192] The carbon units transferred by the coenzymes are present in varying states of reduction. Coenzyme activity appears to be greater with polyglutamate, rather than monoglutamate, forms of folate. Metabolic reactions requiring THF coenzymes include interconversion of serine and glycine; methionine synthesis from homocysteine (also a B_{12}-dependent pathway); histidine degradation to glutamic acid by means of formiminoglutamic

acid (FIGLU); purine biosynthesis; synthesis of the pyrimidine thymidylate, required in DNA synthesis; and the methylation of biogenic amines, including dopamine, tryptamine, serotonin, adrenaline, noradrenaline, and the generation/activation of formate.[42,192] A number of studies have suggested a role for folate in the reversal of preneoplastic conditions of cervical and lung cancers.[255]

Both biochemical and hematologic changes (Table 29-18) are characteristic of poor folate nutriture. The principal clinical feature of folate deficiency is megaloblastic anemia, but folate depletion may precede anemia by months. Other signs and symptoms of deficiency include anorexia, glossitis, nausea, diarrhea, hepatosplenomegaly, and hyperpigmentation of the skin.[42,64] Neurologic disorders also have been attributed to folate deficiency, although this is not routinely part of the clinical picture. Serum folate levels fall below normal after as few as three weeks of folate deprivation.[28] Deficiency of folate leads to inadequate synthesis of DNA and abnormal cell division. Morphologic evidence of the biochemical inadequacy includes bone marrow megaloblastosis, appearance of hypersegmented neutrophils in the peripheral blood, and macrocytosis of reticulocytes and platelets.[95] When red cell folate levels are less than 100 ng/mL (226.6 nmol/L), morphologic abnormalities in mature circulating red blood cells are detected with development of a macrocytic, normoblastic, or megaloblastic anemia. An elevated mean red cell volume and low hemoglobin are consequences of long-standing folate deficiency.[100] Elevation of certain metabolites in the serum serves as an early indicator of suboptimal levels of folate. For example, poor folate status can result in higher plasma levels of the atherogenic amino acid homocysteine.[49,123,180,267] Because of a lack of 5-methyltetrahydrofolate in amounts sufficient for the remethylation of homocysteine to methionine, homocysteine accumulates in the plasma. Toxic effects arising from excess homocysteine may be due to its interference with normal cross linking of collagen molecules, thereby disrupting or damaging the intimal surface of arteries. Low normal serum folate concentrations could therefore place an individual at increased risk of cardiovascular disease. Supplementation with modest doses of folate (1 to 5 mg/d) can often normalize elevated homocysteine concentrations.[25,124,216]

Approximately 6000 infants are born each year in the U.S. with neural tube defects. Maternal folic acid supplementation in early pregnancy reduces the risk of giving birth to an infant with a neural tube defect (*e.g.*, spina bifida or anencephaly) by as much as 75%. Because closure of the embryonic neural tube normally occurs by the sixth week of pregnancy, there is no deterrent advantage reported for women who begin supplementation after that point in time.[160,203,280] Folic acid fortification of basic foods, such as wheat flour, has been advocated. While this action would address the issue of women of child-bearing age receiving the vitamin in amounts sufficient to reduce the risk of fetal neural tube defects, it could create a medical dilemma for the elderly. It is estimated that pernicious anemia (PA) caused by malabsorption of vitamin B_{12} effects approxi-

TABLE 29-18. Sequence of Events in Developing Folate Deficiency. Earliest Abnormalities in Each Stage are Boxed



Serum Folate (ng/ml)	>10	>10	>5	<3	<3	<3	<3
RBC Folate (ng/ml)	>400	>300	>200	>200	<160	<120	<100
Diagnostic dU Suppression	Normal	Normal	Normal	Normal	Normal	Abnormal*	Abnormal*
Lobe Average	<3.5	<3.5	<3.5	<3.5	<3.5	>3.5	>3.5
Liver Folate (μg/g)	>5	>400	>3	>3	<1.6	<1.2	<1
Erythrocytes	Normal	Normal	Normal	Normal	Normal	Normal	Macroovalocytic
MCV	Normal	Normal	Normal	Normal	Normal	Normal	Elevated
Hemoglobin (g/dL)	>12	>12	>12	>12	>12	>12	>12
Plasma Clearance of Intravenous Folate	Normal	Normal	Normal	Normal	Normal	Increased	Increased

**Dietary excess of folate reduces zinc absorption.*

Due to hormonal effects (on receptors?), there may be folate deficiency (i.e. Stage III-IV negative balance) in cervical epithelial cells (a reversible lesion) (possibly precancerous?) when there is only early negative balance (i.e. Stage I-II negative balance) in the erythron (Ran et al. Blood, November 1990).

Source: Herbert B, Das K. Folic acid and vitamin B_{12}. In: Shils ME, Olson JA, Shike M, eds. Modern nutrition in health and disease. 8th ed. Philadelphia: Lea & Febiger, 1994.

mately 1 million Americans. Most of these individuals are older adults. A deficiency of either vitamin B_{12} or folic acid will create the same hematologic picture (*i.e.*, macrocytic, megaloblastic anemia). However, only a B_{12} deficiency will produce irreversible neurologic lesions. Folic acid supplementation can mask or delay diagnosis of B_{12} deficiency by restoring a normal hematologic picture without preventing the B_{12}-induced neurological disease. There is serious concern among health practitioners that widespread fortification of foods with folic acid would significantly increase the incidence of delayed diagnosis of vitamin B_{12} deficiency.[286]

Microbiologic assays of folates in serum, erythrocytes, and urine have been conducted in the clinical laboratory for many years.[110,171] Although not as rapid or convenient as newer radioassay procedures, microbiologic assay remains the reference method. The organism of choice is *Lactobacillus casei* (ATCC 7469), which utilizes all monoglutamate forms of folate, including the reduced form, 5-methyltetrahydrofolate, for growth. RBC folate is present as polyglutamates and must be converted to monoglutamates for analysis. Interference by antibiotics in the patient specimen presents a serious problem.

Indirect measurement of folate status has been attempted by employing a histidine loading test. Histidine is metabolized to glutamic acid by way of the intermediate formiminoglutamic acid (FIGLU). The final enzyme of this pathway, formiminotransferase, is folate-dependent. If folate is deficient, FIGLU accumulates. When an oral 2- to 15-g dose of histidine is administered to a folate-depleted patient, the amount of FIGLU excreted in the urine in the 8-hour period following the load is at least 5 to 10 times greater than the amount excreted by a folate-replete individual under the same conditions.[229]

Another approach in evaluating the adequacy of tissue folate to support normal biochemical function is by means of the deoxyuridine (dU) suppression test, which reflects slowed *de novo* DNA synthesis. The final step in the conversion of deoxyuridylate to thymidylate for DNA synthesis is folate-dependent. This test is generally abnormal in megaloblastic anemia due to both folate and B_{12} deficiency.[36]

There is concern over falsely low serum values for folate arising from oxidative destruction of the vitamin prior to analysis. To avoid vitamin loss, serum may be stored frozen or a reducing substance such as ascorbic acid may be added to the specimen. A recent study on the effect of light on serum folate concluded that specimens to be tested can be stored at room temperature for up to 8 hours in either a gel separator collection tube or in a polypropylene storage tube without substantial loss (<7%) of the vitamin. Folate specimens exposed to light for more than 8 hours should be redrawn.[154] Since folate levels of erythrocytes exceed serum levels by approximately 40-fold, it is essential that hemolyzed samples not be accepted for assay of serum folate. To measure erythrocyte folate levels, a hemolysate, prepared with an aqueous 1% ascorbic acid solution, is tested.[28]

Folate assessment by competitive protein-binding radioassay techniques is common. Tracers used are [^{125}I]folate or ^{3}H-PGA. The weak binding of folate to plasma proteins necessitates pretreatment or a denaturation step to liberate the vitamin before application of CPB techniques. Denaturation may be by heat (boiling) or by pH inactivation (no-boil). Incomplete denaturation of interfering proteins is sometimes experienced with a no-boil protocol. Radioligand assay procedures have been adapted for automated systems to permit simultaneous assays of serum folate and B_{12} after manual heat denaturation of endogenous protein binders.[45] Because these two vitamins are so closely linked in terms of biochemistry and metabolic function, it is important that they be evaluated together.[116]

HPLC is particularly useful in separating the various folate compounds. A competitive enzyme-linked ligand sorbent assay (ELLSA) for quantitation of folates has been described that offers promise for application in the clinical laboratory.[89]

Individuals with a folate deficiency will have a reduced capacity to convert homocysteine to methionine. Measurement of serum levels of homocysteine by modified techniques using capillary-gas chromatography and mass spectrometry have proven useful as a means of identifying suboptimal folate nutriture.[230] Totally automated methods, including a C_{18}-based HPLC assay and an FPIA requiring no pretreatment or chromatographic step, have been reported in the literature recently.[237a]

Vitamin B_{12}

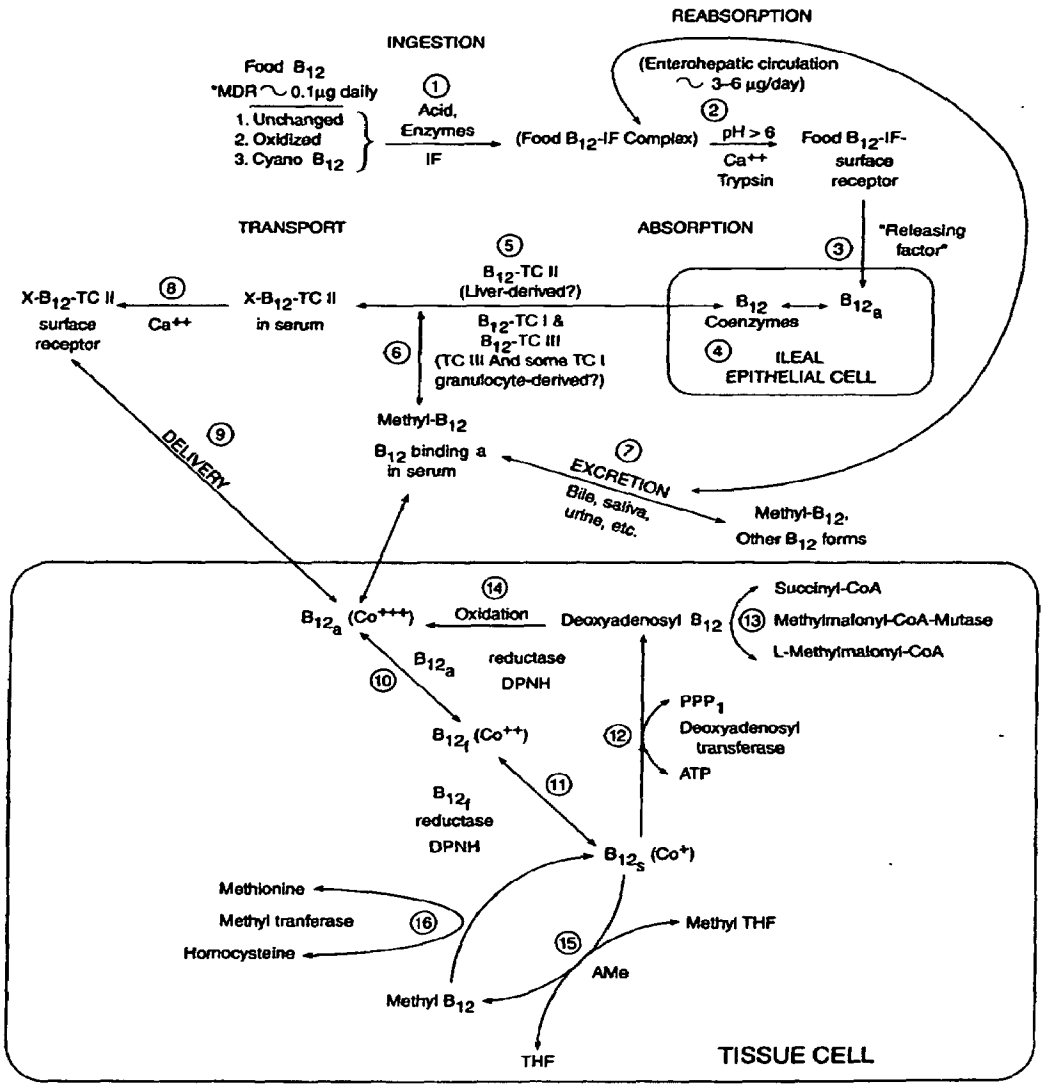
In 1948, vitamin B_{12} was isolated and crystallized for the first time by both American and British researchers.[206] IUPAC recommendations call for generic use of the name *cobalamin* for those vitamins that possess a cobalt-containing corrin ring attached to the nucleotide 5,6-dimethylbenzimidazole (see Table 29-11). Dimethylbenzimidazole is similar in structure to riboflavin. The corrin nucleus contains four substituted pyrrole rings and resembles the porphyrin nucleus of heme. Various ligands may be covalently linked to the cobalt atom, including cyanide anion (cyanocobalamin), hydroxyl group (hydroxocobalamin), methyl group (methylcobalamin), or 5′ deoxyadenosyl group (adenosylcobalamin).[192] The coenzyme forms of B_{12}, adenosylcobalamin and methylcobalamin, function as transmethylating agents.[42] Methylcobalamin accounts for approximately 75% of plasma vitamin B_{12}, whereas a similar percentage of liver B_{12} is in the form of adenosylcobalamin. B_{12} in erythrocytes and the kidney is also largely present as adenosylcobalamin. Smaller amounts of hydroxocobalamin and cyanocobalamin exist in body fluids and tissues.

The RDA for vitamin B_{12} is 2 μg for adults and adolescents of both sexes. In pregnancy and lactation, the requirement is increased, respectively, to 2.2 μg and 2.6 μg daily.[185] Although vegetable matter is devoid of vitamin B_{12}, it is present in animal products such as meat and dairy foods, including liver, eggs, milk, and cheese.[82] Microorganisms alone synthesize the vitamin, and animals, including humans, ultimately depend on this activity to furnish preformed B_{12}. Enteric microorganisms, mainly actinomycetes, synthesize B_{12} in the human colon, but it is not absorbed

through the mucosa in this region of the gastrointestinal tract.[29] B_{12} deficiency is rarely caused by poor nutrition. However, strict vegetarians, unless they receive B_{12} as a contaminant in food or supplement the diet, will develop a clinical deficiency.[96] The liver stores 50% to 90% of the body's B_{12}.[99] Reserves are relatively large, and it may require literally years for the classic features of deficiency to appear, even in complete absence of vitamin intake.

Vitamin B_{12} is absorbed in the intestine, depending primarily on the availability of intrinsic factor (IF), a glycoprotein secreted by gastric parietal cells (Fig. 29-24). These same cells secrete hydrochloric acid. Impaired absorption due to lack of intrinsic factor in gastric secretions gives rise to the clinical condition known as *pernicious anemia (PA)*. Achlorhydria, which diminishes B_{12} absorption, and PA, associated with atrophy of the gastric mucosa, are most common among individuals over 60 years of age. An extremely small percentage of vitamin B_{12}, probably less than 1%, is absorbed passively throughout the intestine, independent of IF complex formation. A diffusion-type mechanism for vitamin uptake, not mediated by IF, also seems to operate when large amounts (100–300 μg) of B_{12} are supplied.

The four common forms of cobalamin bind equally well to IF.[41] In the ileum, IF-B_{12} complex binds to specific membrane receptors of the mucosal brush border. A pH above 6 and the presence of calcium ions are required to promote vitamin absorption. Upon transiting the mucosal cell, vitamin B_{12} is released into the portal circulation. Plasma B_{12} is



* MDR = Adult minimum daily requirement from exogenous sources to sustain normality

▲

Figure 29-24. Flow chart of cobalamin (B_{12}) metabolism. Circled numbers identify individual metabolic steps. *Source:* Herbert B, Das K. Folic acid and vitamin B_{12}. In: Shils ME, Olson JA, Shike M, eds. Modern nutrition in health and disease. 8th ed. Philadelphia: Lea & Febiger, 1994.

bound by members of a group of carrier globulins, the transcobalamins (TC). Transcobalamin II (TCII) serves as primary transport protein for distribution of newly absorbed vitamin B_{12} to the tissues.[99] All cells that synthesize DNA possess surface receptors for TCII. One of the earliest detectable signs of a negative B_{12} balance is reportedly a decrease in serum holotranscobalamin (TCII + cobalamin).[96] Vitamin B_{12} also binds to haptocorrin, a circulating storage protein. The only receptors for haptocorrin are on B_{12} storage cells (*e.g.*, liver and reticuloendothelial cells). Other protein binders of B_{12} have been identified in body fluids, including serum, saliva, tears, milk, colostrum, cerebrospinal fluid, and gastric juice, as well as in blood cells. These endogenous proteins (TCI and TCIII) have been collectively designated as *R proteins* because of their rapid migration during electrophoresis. R proteins bind both biologically active cobalamin and inactive analogs. The physiologic function of these binding proteins is not clear, but they do not facilitate ileal absorption of the vitamin.[231] The therapeutic form of vitamin B_{12} is cyanocobalamin. If it is administered subcutaneously or intramuscularly, the need for IF-mediated intestinal absorption is bypassed. While the treatment of PA with oral B_{12} megadose therapy is more common in Europe than in the U.S., it has proven to be successful.[133] When given orally, in excess, enough B_{12} is absorbed even in the absence of IF to meet the requirements of most patients. Even in megadoses, cobalamin is reported to be nontoxic.

In humans, two enzymes are known to be vitamin B_{12}–dependent: 5-methyltetrahydrofolate (5-methyl-THF) homocysteine methyltransferase and methylmalonylcoenzyme A mutase.[42] Methylcobalamin functions as coenzyme for a methyltransferase reaction in methionine synthesis. The coenzyme form of folate, 5-methyl-THF, donates a methyl group to cobalamin, which transfers it to homocysteine, forming a new amino acid, methionine. Methionine is subsequently metabolized to succinyl-CoA. Thus, both folate and B_{12} participate in methionine synthesis. In the process, tetrahydrofolate (THF), required for synthesis of thymidylate in DNA, is regenerated from 5-methyl-THF. Vitamin B_{12} acts as a methyl receiver to prevent "trapping" of folate as the methylated tetrahydrofolate.[192] Interference with nucleotide synthesis impairs erythropoiesis and leads to development of megaloblastic anemia due either to deficiency of B_{12} or folate. The interrelationship of folate and B_{12} is also seen in a cobalamin requirement for folate uptake by cells.[82] In this instance, a folate deficiency may occur indirectly because of inadequate levels of B_{12}.

Adenosylcobalamin is required by the enzyme methylmalonyl-coenzyme A mutase for rearrangement of 1-methylmalonyl-CoA to succinyl-CoA. Succinyl-CoA is further metabolized through the tricarboxylic acid cycle. In states of B_{12}, but not folate, deficiency, methylmalonyl-CoA is not converted to succinyl-CoA, and methylmalonic acid (MMA) excretion in the urine is increased. In summary, B_{12} functions in oxidative degradation of amino acids and, since methionine is a glycogenic amino acid, in carbohydrate metabolism.[192] Fatty acids with odd numbers of carbon atoms are oxidized by a pathway requiring methylmalonyl-CoA mutase activity. Thus, B_{12} is also essential for normal lipid metabolism.[192] Inadequate supplies of cobalamin will disrupt lipid synthesis. This, along with decreased availability of adenosyl methionine needed for myelin protein formation, could explain the neurologic complications, including demyelination and degeneration of the central nervous system and the optic and peripheral nerves, seen in B_{12} deficiency.

Deficiency of B_{12} may be due to dietary absence, as among strict vegetarians, increased requirements, as in pregnancy, malabsorption due to disease, drug-induced interference (Table 29-19), or intrinsic factor and transport protein inadequacies.[99] Low cobalamin plasma levels are reported in patients with sprue, Crohn's disease, regional enteritis, pernicious anemia, gastric or intestinal resection, multiple myeloma, IF-blocking antibodies, or serum gastric parietal cell autoantibodies. Gastric and intestinal bacterial overgrowth may contribute to cobalamin malabsorption. Up to 25% of the geriatric population may be afflicted with chronic atrophic gastritis. Occurrence of this condition increases with age and may account for the widely reported low serum cobalamin concentrations among the elderly.[2,143,198,263] Studies have shown low serum cobalamin in 10% to 50% of elderly, depending upon variables such as the specific population of older adults examined, assay techniques employed, and cut-off values used to define risk of deficiency. The prevalence of cobalamin deficiency was found to be at least 12% in a large sample of ambulatory older adults when deficiency was defined by a serum cobalamin concentration <258 pmol/L and elevation of one or both of the metabolites MMA and homocysteine. Many elderly with "normal" serum vitamin levels were metabolically deficient in B_{12} or folate.[142] Impaired intestinal absorption of B_{12} has been reported in patients taking anticonvulsants, neomycin, *para*-aminosalicylic acid, phenformin, and cholestyramine, and also has been reported in alcoholics.[27] Controversy continues over reports that megadoses of ascorbic acid may lead to inactivation of vitamin B_{12} and destruction of IF.[27,96]

Clinical features of B_{12} deficiency generally include both hematologic (*e.g.*, macrocytic anemia, megaloblastosis, hypersegmentation of neutrophils) and neurologic (*e.g.*,

TABLE 29-19. Cobalamin-Drug Interactions

Drug	Mechanism of Interaction
Aminosalicylic acid (PAS)	Decreased absorption
Colchicine	Malabsorption
Neomycin	Malabsorption
Guanidines	Decreased absorption
Metformin	Decreased absorption
Phenformin	Decreased absorption
Potassium chloride	Decreased absorption
Nitrous oxide	Interferes with B_{12} metabolism
Fiber	Enhances excretion

Source: Ellenbogen L, Cooper BA. Vitamin B_{12}. In: Machlin LJ, ed. Handbook of Vitamins. 2nd ed. New York: Marcel Dekker, 1991.

peripheral nerve degeneration) manifestations. The hematologic picture is identical in both B_{12} and folate deficiency due to abnormal replication of DNA in hematopoietic tissue. Especially among the elderly, neuropsychiatric disorders may be the primary or only indication of cobalamin deficiency.[141] Numbness, tingling, and weakness of extremities are frequent early neurologic symptoms of vitamin B_{12} deficiency. Vision may be impaired. Spinal cord degeneration leads to changes in tendon reflexes and difficulty in walking. Cognitive dysfunctions include poor memory, loss of mental alertness and confusion, marked personality and mood changes, and, in rare instances, delusions and hallucinations may develop. Research is in progress to determine what, if any, relationship exists among serum cobalamin levels, normal aging, and the occurrence of dementia or Alzheimer's disease.[10,51] Some cognitive and hematopoietic dysfunctions found in AIDS patients have been reversed by vitamin B_{12} therapy. Elevated serum homocysteine concentrations due to vitamin deficiency may play a part since, in excess, the amino acid is both neurotoxic and vasculotoxic.[96]

Limited observations suggest that osteoblast activity depends on cobalamin and that bone metabolism is affected by cobalamin deficiency. Cobalamin-deficient patients were reported to have lower alkaline phosphatase and osteocalcin levels than controls. Osteocalcin, a vitamin K–dependent bone-specific protein, is synthesized by osteoblasts. Its concentration in plasma reflects the rate of bone formation. If so, not only bone marrow cells but also adjoining skeletal cells could be affected in B_{12} deficiency.[37] The osteopenia of aging may be related to an inadequate supply of vitamin B_{12}.[37]

Pernicious anemia (PA), a common cause of vitamin B_{12} deficiency, primarily affects the elderly. Diagnosis of PA by assessment of B_{12} intestinal absorption may be accomplished by measuring urinary excretion of ^{57}Co-labeled vitamin in the Schilling test.[42] An oral dose of ^{57}Co-B_{12} is administered along with a parenteral injection of nonlabeled B_{12}. Labeled B_{12} absorbed in the intestine enters the pool of unlabeled vitamin in the plasma, and both forms are excreted in the urine. The percentage of the oral dose appearing in the urine in 24 hours is calculated. Normal B_{12} absorption is indicated when more than 10% of the oral dose is excreted by the patient. Reduced excretion of radioactive B_{12} is seen in pernicious anemia. If repetition of the test with addition of IF results in increased radioactivity in the urine, lack of functional IF is confirmed. Decreased glomerular filtration, due to either renal disease or aging, and improper urine collection invalidate the test results. With elderly patients, collection and evaluation of a 48-hour urine specimen will improve the accuracy of the test.

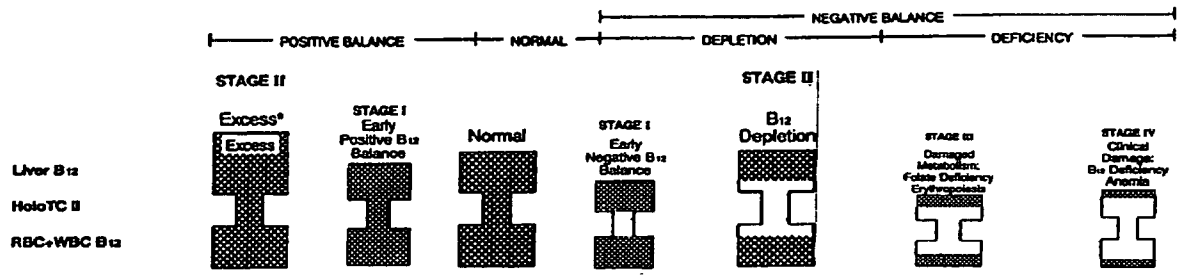
A recent study evaluated the effect of light on serum B_{12} concentrations (111–812 ng/L). Under typical storage conditions encountered in a clinical laboratory, B_{12} was not affected by light for up to 24 hours after collection when stored at room temperature (20–25°C).[154] Depending on the assessment method employed, serum levels of B_{12} range from approximately 200 to 900 pg/mL.[112] B_{12}-deficient erythropoiesis is associated with levels less than 100 pg/mL (74 pmol/L).[66,95] Serum folate and vitamin B_{12} levels must be determined in patients with megaloblastic anemia to pinpoint its etiology. Large-dose folate therapy may bring about transient improvement of megaloblastic anemia associated with B_{12} deficiency, but neurologic damage will develop or progress, often irreversibly. It is essential to distinguish the true nature of the underlying disorder (*e.g.*, folate or B_{12} deficiency) so that appropriate therapy may be provided as quickly as possible.

Some patients with serum B_{12} in the lower portion of the reference range may still develop PA. B_{12} deficiency may be by assessment of serum methylmalonate and homocysteine concentrations (Table 29-20).[95,96,168,245,246] Elevated levels of methylmalonic acid (MMA) and total homocysteine are detected in over 90% of cases of cobalamin deficiency. Measurement of urinary MMA excretion is also diagnostically useful. Increase in these metabolites often occurs before any other clinical evidence of deficiency is manifested. Serum MMA levels >950 nmol/L (110–950 nmol/L) and total homocysteine concentrations >29 micromoles/L (6–29 micromoles/L) indicate B_{12} deficiency even in the presence of normal hematologic parameters. An automated assay of MMA in serum and urine by derivatization with 1-pyrenyldiazomethane, liquid chromatography, and fluorescence detection has recently been described.[233] The risk factor for occlusive atherosclerosis is increased by hyperhomocysteinemia. Improved vitamin B_{12} status normalizes homocysteine levels within weeks, thereby reducing the patient's risk of coronary artery disease.

Cobalamin determinations may be by microbiologic or radioligand assays. Although a variety of vitamin B_{12}–dependent test organisms have been used, including *Euglena gracilis, Lactobacillus leichmannii* (ATCC 7830) remains the microorganism of choice.[79] Microbiological assay is used as the reference method or in a research setting. In the clinical laboratory, radioassays are routinely used for determination of serum B_{12} levels. Differential radioassays measure cobalamin content more accurately than do microbiologic assays, since noncobalamin corrinoids not utilized by humans will support microbial growth.[95] Plasma transcobalamins must be heat denatured (boiling) or subjected to alkaline pH inactivation (no-boil) prior to either microbiologic or radioassay of the specimen to release the cobalamin for measurement.

Radioisotope dilution methods are the most widely used assays for cobalamin. These competitive inhibition radioassays measure the extent to which cobalamin, after being freed from bound materials, competes with radioactive cyanocobalamin for binding sites on a protein.[66] Radioligand assays may be either RIA or CBP procedures. In the case of CBP assays, purified IF has been strongly recommended as the cobalamin-binding protein. A semiautomated radioassay system makes possible simultaneous assessments of serum B_{12} and folate, following off-line denaturation of endogenous binding proteins.[45] Purified IF is used as the competitive binding protein, with solid-phase adsorbent separating free and bound ^{57}Co. Recently, it has been reported that no boiling or other pretreatment of patient specimen is required when a non-intrinsic factor blocking agent is used along with a magnetizable solid-phase separation system.[111] This assay is highly specific for cobalamin. With elimination of a pretreatment requirement, and ease of separation

TABLE 29-20. Sequential Stages of Vitamin B-12 Status. Biochemical and Hematological Sequence of Events as Negative Vitamin B-12 Balance Progresses. [© 1990, 1993 Victor Herbert (Modified 1993 to Include Homocysteine).]



HoloTC II (pg/ml) (in equilibrium with TCII receptors [on DNA-synthesizing cells])	>100	>100	>50	<40	<40	<40	<40
TC II % sat. (Caution: Apo TCII is an acute phase reactant)	>5%	>5%	>5%	<4%	<4%	<4%	<4%
Holohap (pg/ml)¥ (in equilibrium with haptocorrin receptors [on B_{12}-storage cells])	>500	>400	>180	>180	<150 ¥	<100	<100
dU Suppression	Normal	Normal	Normal	Normal	Normal	Abnormal	Abnormal
Hypersegmentation	No	No	No	No	No	Yes	Yes
TBBC† % sat.	>50%	>40	>15%	>15%	>15%	<15%	<10%
Hap % sat.	>50%	>40	>20%	>20%	>20%	<20%	<10%
RBC Folate (ng/mL)	>160	>160	>160	>160	>160	<140	<100
RBC Cobalamin (ng/ml)	<800	<600	300–800	<300	<200	<150	<100
Homocystiene †	No	No	No	No	No	Yes	No
Erythrocytes	Normal	Normal	Normal	Normal	Normal	Normal	Macroovalocytic
MCV	Normal	Normal	Normal	Normal	Normal	Normal	Elevated
Hemoglobin	Normal	Normal	Normal	Normal	Normal	Normal	Low
TC II	Normal	Normal	Normal	Normal	Normal	Elevated	Elevated
Homocysteine and/or Methylmalonate †≠	No	No	No	No	No	?	Yes
Myelin Damage	No*	No	No	No	No	?	Frequent
Holo TC II cell receptors	Normal	Normal	Normal	Up-regulated?	Down-regulated?	Elevated in plasma	

**Cyanocobalamin excesses (injected or intranasal) produce transient rise in B_{12} analogues on B_{12} delivery protein (TC II); the significance of such rises is unknown (Herbert et al., 1987). Cyanocobalamin acts as an anti-B_{12} in a rare congenital defect in B_{12} metabolism.*

≠ In serum and urine.

† TBBC = Total B_{12} binding capacity.

¥ Low holohaptocorrin correlates with liver cell B_{12} depletion. There may be hematopoietic cell and glial cell B_{12} depletion prior to liver cell depletion, and those cells may be in STAGE III or IV negative B_{12} balance while liver cells are still in STAGE II.

achieved in a magnetic radioassay, a fully automated continuous-flow procedure can be realized. Assay automation of B_{12} on the Abbott IM$_x$ provides rapid results in a nonradioisotopic format.[129] B_{12} deficiency can be detected and quantitated by measuring methylmalonic acid in urine or assessing its serum level using capillary gas chromatography/mass spectrometry.[245,246]

SUMMARY

The Joint Commission on Accreditation of Healthcare Organizations (JCAHO) is mandating more stringent nutritional review of all patients. There can be no doubt that this will impact the clinical laboratory. The clinical laboratorian will be required to know more about vitamins, their biochemical functions and physiologic roles, and the best assay methodologies to use to provide the clinician with timely information on the patient's nutritional status. There are financial implications to optimizing a patient's nutritional status, thereby hastening the desired medical outcomes and reducing the patient's length of stay in the hospital.

The general public is also increasingly concerned with health promotion and disease prevention. Supplemental use of vitamins to increase longevity and improve the quality of life is regularly advocated in the media. Vitamin sales is a multi-billion-dollar commercial enterprise in this country. While there is strong support for the beneficial effects of vitamins in the prevention of certain cancers and cardiovascular disease, there is also concern over the possibility of toxicity from overly aggressive vitamin supplementation.[200]

Historically, medicine has focused more attention on conditions of vitamin deficiency than excess. Despite the high standard of living in this country, significant numbers of individuals are characterized by an overall vitamin status that is suboptimal or overtly deficient. Nutritional requirements in special physiological states such as growth, preg-

nancy and lactation, and aging may not be met by dietary consumption. For example, age-related changes in vitamin status due to altered dietary practices, physiologic changes, and drug-nutrient interaction contribute to the risk for deficiency of one or more vitamins among the 32 million Americans who are over 65 years of age.

Biochemical determinations of vitamin status and the monitoring of nutritional support will increase in the years ahead. In the future, vitamin assays will not be viewed as esoteric reference laboratory procedures; rather they will be acknowledged as essential for the promotion of wellness and for the cost-effective provision of quality health care.

CASE STUDY 29-1

During her most recent physical examination, a blood pressure of 175/96 had been recorded for a widowed, 65-year-old female. Over the past 3 years, her blood pressure as recorded on annual physical examinations had gradually risen, but this report was the first clear indication of hypertension. Her physician prescribed 150 mg of hydralazine per day, administered orally. In follow-up office visits, her physician noted that the dosage prescribed was not producing a satisfactory lowering of the patient's blood pressure. Adjustment of dosage was attempted, and satisfactory results were finally achieved with 400 mg of hydralazine daily. Several months after initiation of therapy, the patient's daughter called the physician to report pronounced changes in her mother's personality. Her usual optimism had been replaced by depression and irritability. In addition, her daughter indicated that the woman no longer appeared interested in her house or her family. She was reluctant to cook for herself but had purchased a supply of high-protein supplement, which she consumed for nourishment. Such a lack of responsibility was not in keeping with her mother's traditional behavior. These changes, coupled with the appearance of a rash on her mother's forehead, prompted the daughter to bring her mother to the clinic. Upon review of the medication record and noting signs of peripheral nerve inflammation in the patient, the physician requested the laboratory to evaluate the patient's vitamin B_6 status.

Questions

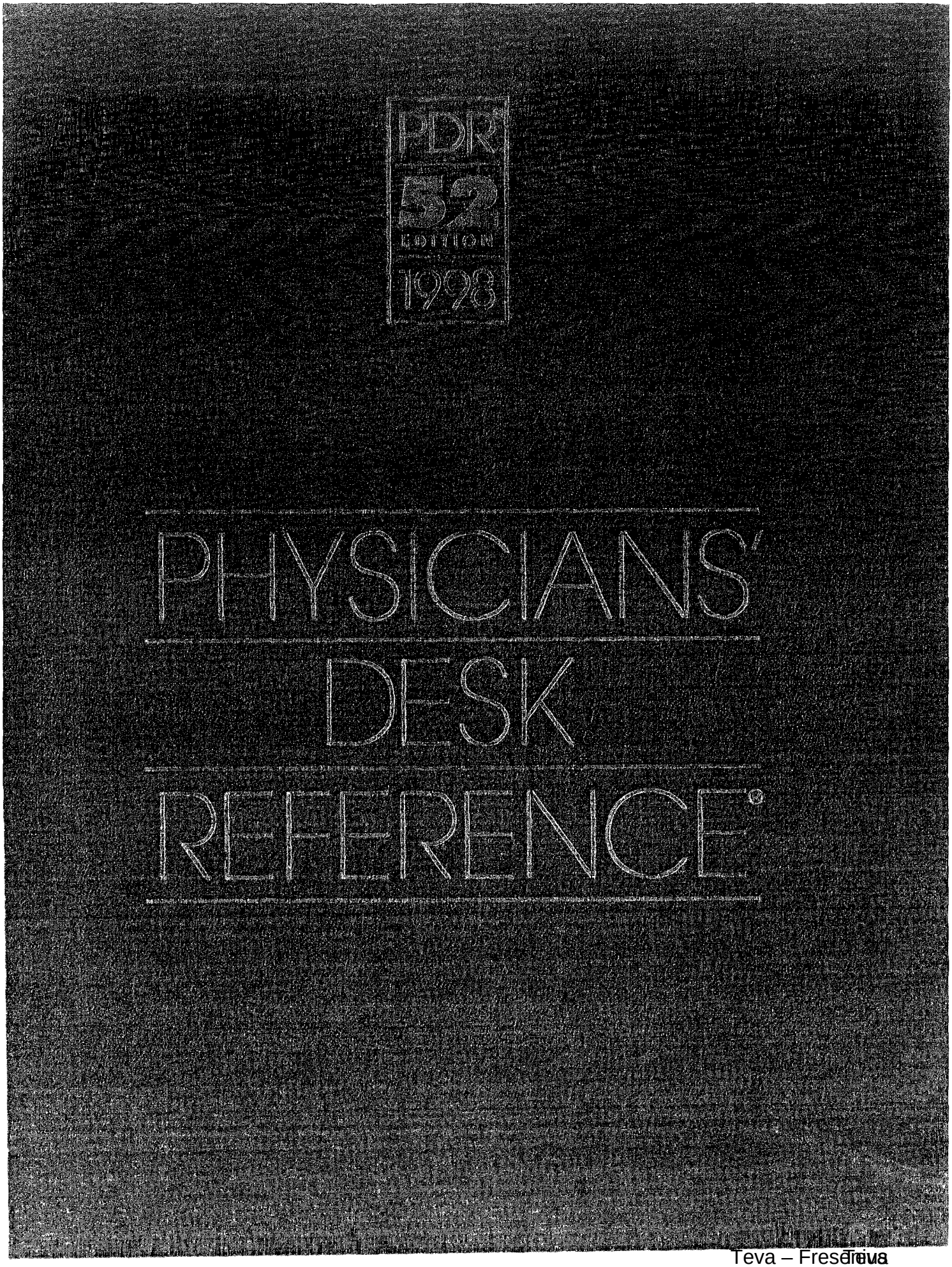
1. What type of assessment procedure will the laboratory be most likely to employ in evaluating the patient's vitamin B_6 status?
2. Identify the patient specimen required for testing and any special precautions to be taken in its handling or processing.
3. What clinical manifestations suggested a vitamin B_6 deficiency to the physician?
4. In what way is it likely that the patient's medication and dietary practices contributed to development of a B_6 deficiency?
5. A marginal or deficient vitamin B_6 status is indicated by laboratory values of what magnitude?

CASE STUDY 29-2

A 62-year-old male had been admitted to the hospital with a diagnosis of acute myocardial infarction. Anticoagulant therapy was initiated in an attempt to reduce the incidence of secondary thromboembolism. While he was hospitalized, heparin therapy had been initiated, and, upon discharge, the patient was switched to Coumadin. For 3 months after leaving the hospital, the patient had been completely stable on a Coumadin regimen of 30 mg per week. During a follow-up visit to his physician, the man's prothrombin time was reported as 12 seconds, as compared with previously obtained PT times of 22 to 24 seconds. Effective oral anticoagulant therapy calls for maintenance of a prothrombin time that exceeds "normal" by 1.5 to 1.7 times. Review of the patient's medication record did not suggest drug interference as the basis for the decreased anticoagulant effect. A careful dietary history provided an explanation for the newly acquired warfarin resistance.

Questions

1. Excessive intake of what vitamin is likely to account for the observed shortening of prothrombin time?
2. Describe the physiologic function of this vitamin.
3. Suggest possible dietary practices that could induce warfarin (Coumadin) resistance.



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FLUOROURACIL R

flu 'ro-u 'ra-sil]

INJECTION

The following text is complete prescribing information based on official labeling in effect June 1997.

WARNING

It is recommended that FLUOROURACIL be given only by or under the supervision of a qualified physician who is experienced in cancer chemotherapy and who is well versed in the use of potent antimetabolites. Because of the possibility of severe toxic reactions, it is recommended that patients be hospitalized at least during the initial course of therapy.

DESCRIPTION

FLUOROURACIL INJECTION, an antineoplastic antimetabolite, is a sterile, nonpyrogenic injectable solution for intravenous administration. Each 10-mL contains 500 mg fluorouracil; pH is adjusted to approximately 9.2 with sodium hydroxide.

Chemically, fluorouracil, a fluorinated pyrimidine, is 5-fluoro-2,4 (1*H*,3*H*)-pyrimidinedione. It is a white to practically white crystalline powder which is sparingly soluble in water. The molecular weight of fluorouracil is 130.08.

CLINICAL PHARMACOLOGY

There is evidence that the metabolism of fluorouracil in the anabolic pathway blocks the methylation reaction of deoxyuridylic acid to thymidylic acid. In this manner, fluorouracil interferes with the synthesis of deoxyribonucleic acid (DNA) and to a lesser extent inhibits the formation of ribonucleic acid (RNA). Since DNA and RNA are essential for cell division and growth, the effect of fluorouracil may be to create a thymine deficiency which provokes unbalanced growth and death of the cell. The effects of DNA and RNA deprivation are most marked on those cells which grow more rapidly and which take up fluorouracil at a more rapid rate.

Following intravenous injection, fluorouracil distributes into tumors, intestinal mucosa, bone marrow, liver and other tissues throughout the body. In spite of its limited lipid solubility, fluorouracil diffuses readily across the blood-brain barrier and distributes into cerebrospinal fluid and brain tissue.

Seven percent to 20% of the parent drug is excreted unchanged in the urine in 6 hours; of this over 90% is excreted in the first hour. The remaining percentage of the administered dose is metabolized, primarily in the liver. The catabolic metabolism of fluorouracil results in degradation products (eg, CO_2, urea and α-fluoro-β-alanine) which are inactive. The inactive metabolites are excreted in the urine over the next 3 to 4 hours. When fluorouracil is labeled in the six carbon position, thus preventing the ^{14}C metabolism to CO_2, approximately 90% of the total radioactivity is excreted in the urine. When fluorouracil is labeled in the two carbon position approximately 90% of the total radioactivity is excreted in expired CO_2. Ninety percent of the dose is accounted for during the first 24 hours following intravenous administration.

Following intravenous administration of fluorouracil, the mean half-life of elimination from plasma is approximately 16 minutes, with a range of 8 to 20 minutes, and is dose dependent. No intact drug can be detected in the plasma 3 hours after an intravenous injection.

INDICATIONS AND USAGE

Fluorouracil is effective in the palliative management of carcinoma of the colon, rectum, breast, stomach and pancreas.

CONTRAINDICATIONS

Fluorouracil therapy is contraindicated for patients in a poor nutritional state, those with depressed bone marrow function, those with potentially serious infections or those with a known hypersensitivity to Fluorouracil.

WARNINGS

THE DAILY DOSE OF FLUOROURACIL IS NOT TO EXCEED 800 MG. IT IS RECOMMENDED THAT PATIENTS BE HOSPITALIZED DURING THEIR FIRST COURSE OF TREATMENT.

Fluorouracil should be used with extreme caution in poor risk patients with a history of high-dose pelvic irradiation or previous use of alkylating agents, those who have a widespread involvement of bone marrow by metastatic tumors or those with impaired hepatic or renal function.

Rarely, unexpected, severe toxicity (eg, stomatitis, diarrhea, neutropenia and neurotoxicity) associated with 5-FU has been attributed to deficiency of dipyrimidine dehydrogenase activity.[1] A few patients have been rechallenged with 5-FU and despite 5-FU dose lowering, toxicity recurred and progressed with worse morbidity. Absence of this catabolic enzyme appears to result in prolonged clearance of 5-FU.

Pregnancy: Teratogenic Effects: Pregnancy Category D. Fluorouracil may cause fetal harm when administered to a pregnant woman. Fluorouracil has been shown to be teratogenic in laboratory animals. Fluorouracil exhibited maximum teratogenicity when given to mice as single intraperitoneal injections of 10 to 40 mg/kg on day 10 or 12 of gestation. Similarly, intraperitoneal doses of 12 to 37 mg/kg given to rats between days 9 and 12 of gestation and intramuscular doses of 3 to 9 mg given to hamsters between days 8 and 11 of gestation were teratogenic. Malformations included cleft palates, skeletal defects and deformed appendages, paws and tails. The dosages which were teratogenic in animals are 1 to 3 times the maximum recommended human therapeutic dose. In monkeys, divided doses of 40 mg/kg given between days 20 and 24 of gestation were not teratogenic.

There are no adequate and well-controlled studies with Fluorouracil in pregnant women. While there is no evidence of teratogenicity in humans due to Fluorouracil, it should be kept in mind that other drugs which inhibit DNA synthesis (eg, methotrexate and aminopterin) have been reported to be teratogenic in humans. Women of childbearing potential should be advised to avoid becoming pregnant. If the drug is used during pregnancy, or if the patient becomes pregnant while taking the drug, the patient should be told of the potential hazard to the fetus. Fluorouracil should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Combination Therapy: Any form of therapy which adds to the stress of the patient, interferes with nutrition or depresses bone marrow function will increase the toxicity of Fluorouracil.

Rarely, unexpected, severe toxicity (eg, stomatitis, diarrhea, neutropenia and neurotoxicity) associated with 5-FU has been attributed to deficiency of dipyrimidine dehydrogenase activity.[1] A few patients have been rechallenged with 5-FU and despite 5-FU dose lowering, toxicity recurred and progressed with worse morbidity. Absence of this catabolic enzyme appears to result in prolonged clearance of 5-FU.

PRECAUTIONS

General: Fluorouracil is a highly toxic drug with a narrow margin of safety. Therefore, patients should be carefully supervised, since therapeutic response is unlikely to occur without some evidence of toxicity. Severe hematological toxicity, gastrointestinal hemorrhage and even death may result from the use of Fluorouracil despite meticulous selection of patients and careful adjustment of dosage. Although severe toxicity is more likely in poor risk patients, fatalities may be encountered occasionally even in patients in relatively good condition.

Therapy is to be discontinued promptly whenever one of the following signs of toxicity appears:

Stomatitis or esophagopharyngitis, at the first visible sign.

Leukopenia (WBC under 3500) or a rapidly falling white blood count.

Vomiting, intractable.

Diarrhea, frequent bowel movements or watery stools.

Gastrointestinal ulceration and bleeding.

Thrombocytopenia (platelets under 100,000).

Hemorrhage from any site.

The administration of 5-fluorouracil has been associated with the occurrence of palmar-plantar erythrodysesthesia syndrome, also known as hand-foot syndrome. This syndrome has been characterized as a tingling sensation of hands and feet which may progress over the next few days to pain when holding objects or walking. The palms and soles become symmetrically swollen and erythematous with tenderness of the distal phalanges, possibly accompanied by desquamation. Interruption of therapy is followed by gradual resolution over 5 to 7 days. Although pyridoxine has been reported to ameliorate the palmar-plantar erythrodysesthesia syndrome, its safety and effectiveness have not been established.

Information for Patients: Patients should be informed of expected toxic effects, particularly oral manifestations. Patients should be alerted to the possibility of alopecia as a result of therapy and should be informed that it is usually a transient effect.

Laboratory Tests: White blood counts with differential are recommended before each dose.

Drug Interactions: Leucovorin calcium may enhance the toxicity of fluorouracil.

Also see WARNINGS section.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Carcinogenesis: Long-term studies in animals to evaluate the carcinogenic potential of fluorouracil have not been conducted. However, there was no evidence of carcinogenicity in small groups of rats given fluorouracil orally at doses of 0.01, 0.3, 1 or 3 mg per rat 5 days per week for 52 weeks, followed by a 6-month observation period. Also, in other studies, 33 mg/kg of flourouracil was administered intravenously to male rats once a week for 52 weeks followed by observation for the remainder of their lifetimes with no evidence of carcinogenicity. Female mice were given 1 mg of fluorouracil intravenously once a week for 16 weeks with no effect on the incidence of lung adenomas. On the basis of the available data, no evaluation can be made of the carcinogenic risk of fluorouracil to humans.

Mutagenesis: Oncogenic transformation of fibroblasts from mouse embryo has been induced in vitro by fluorouracil, but the relationship between oncogenicity and mutagenicity is not clear. Fluorouracil has been shown to be mutagenic to several strains of *Salmonella typhimurium,* including TA 1535, TA 1537 and TA 1538, and to *Saccharomyces cerevisiae,* although no evidence of mutagenicity was found with *Salmonella typhimurium* strains TA 92, TA 98 and TA 100. In addition, a positive effect was observed in the micronucleus test on bone marrow cells of the mouse, and fluorouracil at very high concentrations produced chromosomal breaks in hamster fibroblasts in vitro.

Impairment of Fertility: Fluorouracil has not been adequately studied in animals to permit an evaluation of its effects on fertility and general reproductive performance. However, doses of 125 or 250 mg/kg, administered intraperitoneally, have been shown to induce chromosomal aberrations and changes in chromosomal organization of spermatogonia in rats. Spermatogonial differentiation was also inhibited by fluorouracil, resulting in transient infertility. However, in studies with a strain of mouse which is sensitive to the induction of sperm head abnormalities after exposure to a range of chemical mutagens and carcinogens, fluorouracil did not produce any abnormalities at oral doses of up to 80 mg/kg/day. In female rats, fluorouracil, administered intraperitoneally at weekly doses of 25 or 50 mg/kg for 3 weeks during the pre-ovulatory phases of oogenesis, significantly reduced the incidence of fertile matings, delayed the development of pre- and post-implantation embryos, increased the incidence of pre-implantation lethality and induced chromosomal anomalies in these embryos. In a limited study in rabbits, a single 25 mg/kg dose of fluorouracil or 5 daily doses of 5 mg/kg had no effect on ovulation, appeared not to affect implantation and had only a limited effect in producing zygote destruction. Compounds such as fluorouracil, which interfere with DNA, RNA and protein synthesis, might be expected to have adverse effects on gametogenesis.

Pregnancy: Pregnancy Category D. See WARNINGS section.

Nonteratogenic Effects: Fluorouracil has not been studied in animals for its effects on peri- and postnatal development. However, fluorouracil has been shown to cross the placenta and enter into fetal circulation in the rat. Administration of fluorouracil has resulted in increased resorptions and embryolethality in rats. In monkeys, maternal doses higher than 40 mg/kg resulted in abortion of all embryos exposed to fluorouracil. Compounds which inhibit DNA, RNA and protein synthesis might be expected to have adverse effects on peri- and postnatal development.

Nursing Mothers: It is not known whether fluorouracil is excreted in human milk. Because fluorouracil inhibits DNA, RNA and protein synthesis, mothers should not nurse while receiving this drug.

Pediatric Use: Safety and effectiveness in children have not been established.

ADVERSE REACTIONS

Stomatitis and esophagopharyngitis (which may lead to sloughing and ulceration), diarrhea, anorexia, nausea and emesis are commonly seen during therapy.

Leukopenia usually follows every course of adequate therapy with Fluorouracil. The lowest white blood cell counts are commonly observed between the 9th and 14th days after the first course of treatment, although uncommonly the maximal depression may be delayed for as long as 20 days. By the 30th day the count has usually returned to the normal range.

Alopecia and dermatitis may be seen in a substantial number of cases. The dermatitis most often seen is a pruritic maculopapular rash usually appearing on the extremities and less frequently on the trunk. It is generally reversible and usually responsive to symptomatic treatment.

Other adverse reactions are:

Hematologic: pancytopenia, thrombocytopenia, agranulocytosis, anemia.

Cardiovascular: myocardial ischemia, angina.

Gastrointestinal: gastrointestinal ulceration and bleeding.

Allergic Reactions: anaphylaxis and generalized allergic reactions.

Neurologic: acute cerebellar syndrome (which may persist following discontinuance of treatment), nystagmus, headache.

Dermatologic: dry skin; fissuring; photosensitivity, as manifested by erythema or increased pigmentation of the

Continued on next page

Fluorouracil—Cont.

skin; vein pigmentation, palmar-plantar erythrodysesthesia syndrome, as manifested by tingling of the hands and feet followed by pain, erythema and swelling.
Ophthalmic: lacrimal duct stenosis, visual changes, lacrimation, photophobia.
Psychiatric: disorientation, confusion, euphoria.
Miscellaneous: thrombophlebitis, epistaxis, nail changes including loss of nails.

OVERDOSAGE

The possibility of overdosage with Fluorouracil is unlikely in view of the mode of administration. Nevertheless, the anticipated manifestations would be nausea, vomiting, diarrhea, gastrointestinal ulceration and bleeding, bone marrow depression (including thrombocytopenia, leukopenia and agranulocytosis). No specific antidotal therapy exists. Patients who have been exposed to an overdose of Fluorouracil should be monitored hematologically for at least four weeks. Should abnormalities appear, appropriate therapy should be utilized.

The acute intravenous toxicity of fluorouracil is as follows:

Species	LD_{50} (mg/kg±S.E.)
Mouse	340 ±17
Rat	165 ±26
Rabbit	27 ±5.1
Dog	31.5 ±3.8

DOSAGE AND ADMINISTRATION

General Instructions: Fluorouracil Injection should be administered only intravenously, using care to avoid extravasation. No dilution is required.

All dosages are based on the patient's actual weight. However, the estimated lean body mass (dry weight) is used if the patient is obese or if there has been a spurious weight gain due to edema, ascites or other forms of abnormal fluid retention.

It is recommended that prior to treatment each patient be carefully evaluated in order to estimate as accurately as possible the optimum initial dosage of Fluorouracil.

Dosage: 12 mg/kg are given intravenously once daily for 4 successive days. The daily dose should not exceed 800 mg. *If no toxicity is observed,* 6 mg/kg are given on the 6th, 8th, 10th and 12th days *unless toxicity occurs.* No therapy is given on the 5th, 7th, 9th or 11th days. *Therapy is to be discontinued at the end of the 12th day, even if no toxicity has become apparent.* (See WARNINGS and PRECAUTIONS sections.)

Poor risk patients or those who are not in an adequate nutritional state (see CONTRAINDICATIONS and WARNINGS sections) should receive 6 mg/kg/day for 3 days. *If no toxicity is observed,* 3 mg/kg may be given on the 5th, 7th and 9th days *unless toxicity occurs.* No therapy is given on the 4th, 6th or 8th days. The daily dose should not exceed 400 mg.

A sequence of injections on either schedule constitutes a "course of therapy."

Maintenance Therapy: In instances where toxicity has not been a problem, it is recommended that therapy be continued using either of the following schedules:

1. Repeat dosage of first course every 30 days after the last day of the previous course of treatment.
2. When toxic signs resulting from the initial course of therapy have subsided, administer a maintenance dosage of 10 to 15 mg/kg/week as a single dose. Do not exceed 1 gm per week.

The patient's reaction to the previous course of therapy should be taken into account in determining the amount of the drug to be used, and the dosage should be adjusted accordingly. Some patients have received from 9 to 45 courses of treatment during periods which ranged from 12 to 60 months.

Procedures for proper handling and disposal of anticancer drugs should be considered. Several guidelines on this subject have been published.[2-7] There is no general agreement that all of the procedures recommended in the guidelines are necessary or appropriate.

Note: Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. Although the Fluorouracil solution may discolor slightly during storage, the potency and safety are not adversely affected. If a precipitate occurs due to exposure to low temperatures, resolubilize by heating to 140°F and shaking vigorously; allow to cool to body temperature before using.

HOW SUPPLIED

For intravenous use—10-mL single-use vials, boxes of 10 (NDC 0004-1977-01). Each 10 mL contains 500 mg fluorouracil in a colorless to faint yellow aqueous solution, with pH adjusted to approximately 9.2 with sodium hydroxide.

Store at room temperature (59° to 86°F; 15° to 30°C). Protect from light.

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Revised: August 1994

STERILE **R**

FUDR

[ef-u-dee-are]

brand of floxuridine

The following text is complete prescribing information based on official labeling in effect June 1997.

WARNING

It is recommended that FUDR be given only by or under the supervision of a qualified physician who is experienced in cancer chemotherapy and intra-arterial drug therapy and is well versed in the use of potent antimetabolites.

Because of the possibility of severe toxic reactions, all patients should be hospitalized for initiation of the first course of therapy.

DESCRIPTION

Sterile FUDR (floxuridine), an antineoplastic antimetabolite, is available as a sterile, nonpyrogenic, lyophilized powder for reconstitution. Each vial contains 500 mg of floxuridine which is to be reconstituted with 5 mL of sterile water for injection. An appropriate amount of reconstituted solution is then diluted with a parenteral solution for intra-arterial infusion (see DOSAGE AND ADMINISTRATION section).

Floxuridine is a fluorinated pyrimidine. Chemically, floxuridine is 2'-deoxy-5-fluorouridine with an empirical formula of $C_9H_{11}FN_2O_5$. It is a white to off-white odorless solid which is freely soluble in water.

The 2% aqueous solution has a pH of between 4.0 to 5.5. The molecular weight of floxuridine is 246.19.

CLINICAL PHARMACOLOGY

When FUDR is given by rapid intra-arterial injection it is apparently rapidly catabolized to 5-fluorouracil. Thus, rapid injection of FUDR produces the same toxic and antimetabolic effects as does 5-fluorouracil. The primary effect is to interfere with the synthesis of deoxyribonucleic acid (DNA) and to a lesser extent inhibit the formation of ribonucleic acid (RNA). However, when FUDR is given by continuous intra-arterial infusion its direct anabolism to FUDR-monophosphate is enhanced, thus increasing the inhibition of DNA.

Floxuridine is metabolized in the liver. The drug is excreted intact and as urea, fluorouracil, α-fluoro-β-ureidopropionic acid, dihydrofluorouracil, α-fluoro-β-guanidopropionic acid and α-fluoro-β-alanine in the urine; it is also expired as respiratory carbon dioxide. Pharmacokinetic data on intra-arterial infusion of FUDR are not available.

INDICATIONS AND USAGE

FUDR is effective in the palliative management of gastrointestinal adenocarcinoma metastatic to the liver, when given by continuous regional intra-arterial infusion in carefully selected patients who are considered incurable by surgery or other means. Patients with known disease extending beyond an area capable of infusion via a single artery should, except in unusual circumstances, be considered for systemic therapy with other chemotherapeutic agents.

CONTRAINDICATIONS

FUDR therapy is contraindicated for patients in a poor nutritional state, those with depressed bone marrow function or those with potentially serious infections.

WARNINGS

BECAUSE OF THE POSSIBILITY OF SEVERE TOXIC REACTIONS, ALL PATIENTS SHOULD BE HOSPITALIZED FOR THE FIRST COURSE OF THERAPY.

FUDR should be used with extreme caution in patients with impaired hepatic or renal function or following high-dose pelvic irradiation or previous use of alkylating agents. The drug is not intended as an adjuvant.

FUDR may cause fetal harm when administered to a pregnant woman. It has been shown to be teratogenic in the chick embryo, mouse (at doses of 2.5 to 100 mg/kg) and rat (at doses of 75 to 150 mg/kg). Malformations included cleft palates; skeletal defects; and deformed appendages such as paws and tails. The dosages which were teratogenic in animals are 4.2 to 125 times the recommended human therapeutic dose.

There are no adequate and well-controlled studies with FUDR in pregnant women. If this drug is used during pregnancy or if the patient becomes pregnant while receiving this drug, the patient should be apprised of the potential hazard to the fetus. Women of childbearing potential should be advised to avoid becoming pregnant.

Combination Therapy: Any form of therapy which adds on the stress of the patient, interferes with nutrition or depresses bone marrow function will increase the toxicity of FUDR.

PRECAUTIONS

General: Sterile FUDR is a highly toxic drug with a narrow margin of safety. Therefore, patients should be carefully supervised since therapeutic response is unlikely to occur without some evidence of toxicity. Severe hematological toxicity, gastrointestinal hemorrhage and even death may result from the use of FUDR despite meticulous selection of patients and careful adjustment of dosage. Although severe toxicity is more likely in poor risk patients, fatalities may be encountered occasionally even in patients in relatively good condition.

Therapy is to be discontinued promptly whenever one of the following signs of toxicity appears:

Myocardial ischemia
Stomatitis or esophagopharyngitis, at the first visible sign
Leukopenia (WBC under 3500) or a rapidly falling white blood count
Vomiting, intractable
Diarrhea, frequent bowel movements or watery stools
Gastrointestinal ulceration and bleeding
Thrombocytopenia (platelets under 100,000)
Hemorrhage from any site

Information For Patients: Patients should be informed of expected toxic effects, particularly oral manifestations. Patients should be alerted to the possibility of alopecia as a result of therapy and should be informed that it is usually a transient effect.

Laboratory Tests: Careful monitoring of the white blood count and platelet count is recommended.

Drug Interactions: See WARNINGS section.

Carcinogenesis, Mutagenesis, Impairment Of Fertility

Carcinogenesis: Long-term studies in animals to evaluate the carcinogenic potential of floxuridine have not been conducted. On the basis of the available data, no evaluation can be made of the carcinogenic risk of FUDR to humans.

Mutagenesis: Oncogenic transformation of fibroblasts from mouse embryo has been induced in vitro by FUDR, but the relationship between oncogenicity and mutagenicity is not clear. Floxuridine has also been shown to be mutagenic in human leukocytes in vitro and in the *Drosophila* test system. In addition, 5-fluorouracil, to which floxuridine is catabolized when given by intra-arterial injection, has been shown to be mutagenic in in vitro tests.

Impairment Of Fertility: The effects of floxuridine on fertility and general reproductive performance have not been studied in animals. However, because floxuridine is catabolized to 5-fluorouracil, it should be noted that 5-fluorouracil has been shown to induce chromosomal aberrations and changes in chromosome organization of spermatogonia in rats at doses of 125 or 250 mg/kg, administered intraperitoneally.

Spermatogonial differentiation was also inhibited by fluorouracil, resulting in transient infertility. In female rats, fluorouracil, administered intraperitoneally at doses of 25 or 50 mg/kg during the preovulatory phase of oogenesis, significantly reduced the incidence of fertile matings, delayed the development of pre- and post-implantation embryos, increased the incidence of preimplantation lethality and induced chromosomal anomalies in these embryos. Compounds such as FUDR, which interfere with DNA, RNA and protein synthesis, might be expected to have adverse effects on gametogenesis.

Pregnancy: Teratogenic Effects: Pregnancy category D. See WARNINGS section. Floxuridine has been shown to be teratogenic in the chick embryo, mouse (at doses of 2.5 to 100 mg/kg) and rat (at doses of 75 to 150 mg/kg). Malformations included cleft palates, skeletal defects and deformed

[CANCER RESEARCH 57, 1116–1123, March 15, 1997]

LY231514, a Pyrrolo[2,3-d]pyrimidine-based Antifolate That Inhibits Multiple Folate-requiring Enzymes

Chuan Shih,[1] Victor J. Chen, Lynn S. Gossett, Susan B. Gates, Warren C. MacKellar, Lillian L. Habeck, Katherine A. Shackelford, Lurane G. Mendelsohn, Daniel J. Soose, Vinod F. Patel, Sherri L. Andis, Jesse R. Bewley, Elizabeth A. Rayl, Barbara A. Moroson, G. Peter Beardsley, William Kohler, Manshan Ratnam, and Richard M. Schultz

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285 [C. S., V. J. C., L. S. G., S. B. G., W. C. M., L. L. H., K. A. S., L. G. M., D. J. S., V. F. P., S. L. A., J. R. B., R. M. S.]; Department of Pediatrics, Yale University, New Haven, Connecticut 06510 [E. A. R., B. A. M., G. P. B.]; and Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, Ohio 43699 [W. K., M. R.]

ABSTRACT

***N*-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)-ethyl]-benzoyl]-L-glutamic acid (LY231514) is a novel pyrrolo[2,3-d]pyrimidine-based antifolate currently undergoing extensive Phase II clinical trials. Previous studies have established that LY231514 and its synthetic γ-polyglutamates (glu_3 and glu_5) exert potent inhibition against thymidylate synthase (TS). We now report that LY231514 and its polyglutamates also markedly inhibit other key folate-requiring enzymes, including dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltransferase (GARFT). For example, the K_i values of the pentaglutamate of LY231514 are 1.3, 7.2, and 65 nM for inhibition against TS, DHFR, and GARFT, respectively. In contrast, although a similar high level of inhibitory potency was observed for the parent monoglutamate against DHFR (7.0 nM), the inhibition constants (K_i) for the parent monoglutamate are significantly weaker for TS (109 nM) and GARFT (9,300 nM). The effects of LY231514 and its polyglutamates on aminoimidazole carboxamide ribonucleotide formyltransferase, 5,10-methylenetetrahydrofolate dehydrogenase, and 10-formyltetrahydrofolate synthetase were also evaluated. The end product reversal studies conducted in human cell lines further support the concept that multiple enzyme-inhibitory mechanisms are involved in cytotoxicity. The reversal pattern of LY231514 suggests that although TS may be a major site of action for LY231514 at concentrations near the IC_{50}, higher concentrations can lead to inhibition of DHFR and/or other enzymes along the purine *de novo* pathway. Studies with mutant cell lines demonstrated that LY231514 requires polyglutamation and transport via the reduced folate carrier for cytotoxic potency. Therefore, our data suggest that LY231514 is a novel classical antifolate, the antitumor activity of which may result from simultaneous and multiple inhibition of several key folate-requiring enzymes via its polyglutamated metabolites.**

INTRODUCTION

Several novel folate-based antimetabolites are currently being actively investigated in clinical trials. These include lometrexol and LY309887,[2] which inhibit GARFT in the purine *de novo* biosynthetic pathway (1–3); edatrexate (4, 5) which acts on DHFR; and ZD1694 (Tomudex; Refs. 6 and 7), AG337 (Thymitaq; Ref. 8), and BW1843U89 (9), which specifically target TS.

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[1] To whom requests for reprints should be addressed, at Cancer Research Division, Lilly Research Laboratories, Drop 0540, Eli Lilly and Company, 307 E. McCarty St., Indianapolis, IN 46285. Phone: (317) 276–3520; Fax: (317) 277–3652.

[2] The abbreviations used are: LY231514, *N*-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]-benzoyl]-L-glutamic acid; r, recombinant; h, human; m, murine; TS, thymidylate synthase (EC 2.1.1.45); DHFR, dihydrofolate reductase (EC 1.5.1.3); GARFT, glycinamide ribonucleotide formyltransferase (EC 2.1.2.2); AICA, 5-aminoimidazole-4-carboxamide; AICARFT, aminoimidazole carboxamide ribonucleotide formyltransferase (EC 2.1.2.3); C1-S, C1 tetrahydrofolate synthase; FPGS, folylpolyglutamate synthetase (EC 6.3.2.17); RFC, reduced folate carrier; FBP-α, folate binding protein-α; MTT, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; NADPH, β-$NADP^+$, reduced form; 6R-MTHF, 6[R]-5,10-methylene-5,6,7,8-tetrahydrofolate.

LY231514 is a structurally novel antifolate antimetabolite that possesses the unique 6-5-fused pyrrolo[2,3-d]pyrimidine nucleus (10, 11) instead of the more common 6-6-fused pteridine or quinazoline ring structure (Fig. 1). Previous studies have demonstrated that LY231514 is one of the best substrates that is known for the enzyme FPGS (K_m = 1.6 μM and V_{max}/K_m = 621; Ref. 12). It is likely that polyglutamation and the polyglutamated metabolites of LY231514 play profound roles in determining both the selectivity and the antitumor activity of this novel agent (11, 12). Whereas LY231514 only moderately inhibited TS (K_i = 340 nM, recombinant mouse), the pentaglutamate of LY231514 was 100-fold more potent (K_i = 3.4 nM; Ref. 11), making LY231514 one of the most potent folate-based TS inhibitors known today (13).

Preliminary cell culture end product reversal studies in human CCRF-CEM and murine L1210 leukemia cells have demonstrated that thymidine (5 μM) alone was not able to fully reverse the cytotoxic action of LY231514 (11). Both thymidine (5 μM) and hypoxanthine (100 μM) were required to fully protect cells from the growth-inhibitory activity exerted by LY231514. This reversal pattern is significantly different from other TS inhibitors, such as ZD1694 (6) and BW1843U89 (9). Cell culture experiments showed that the antiproliferative activity of LY231514 was completely reversed by the addition of leucovorin (0.05–16 μM) in a competitive manner (11), suggesting that LY231514 competed with natural reduced folate cofactors both at transport and intracellular folate levels and acted as a pure folate antagonist.

Promising antitumor responses have recently been observed in the Phase I trials of LY231514. Moreover, patients who had previously failed to respond to ZD1694 and 5-fluorouracil/leucovorin treatment responded to LY231514 (14). This pattern of clinical response, together with the aforementioned observations of partial protection by thymidine in cell culture, suggest that inhibition of TS by LY231514 may not solely account for the overall antitumor effect of this novel antifolate. LY231514 and its polyglutamates may inhibit other folate-requiring enzymes, such as DHFR, or enzymes along the *de novo* purine biosynthetic pathway. LY231514 may thus act as a multitargeted antifolate, with multiple mechanisms of action affecting the intracellular folate pools and cellular pyrimidine/purine biosynthesis.

We now summarize our findings of LY231514 and its polyglutamates (glu_3 and glu_5) against various folate-requiring enzymes, including human TS, DHFR, AICARFT, 5,10-methylenetetrahydrofolate dehydrogenase, and 10-formyltetrahydrofolate synthetase activities of C1-S and murine GARFT. In addition, we report a detailed comparison of cell culture reversal patterns observed in several human cell lines between compounds LY231514 and ZD1694. Finally, we examine the role of polyglutamation and transport (via the RFC) in the cytotoxicity of LY231514.

MATERIALS AND METHODS

Materials. LY231514 and ZD1694 were prepared according to published methods and procedures (7, 11). The syntheses of the γ-glutamyl derivatives of LY231514 were by the method of Pawelczak *et al.* (15). For *in vitro* studies,

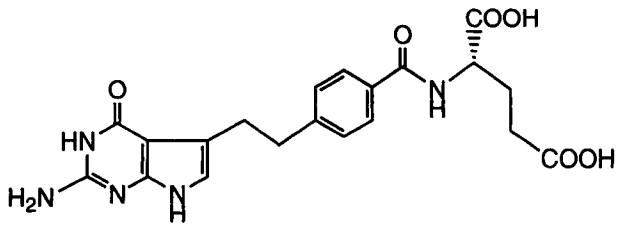

Fig. 1. Structure of LY231514. *N*-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]-benzoyl]-L-glutamic acid.

compounds were dissolved in either DMSO or 5% sodium bicarbonate at an initial concentration of 1–50 mM, and dilutions were made in either enzyme assay buffer or cell culture medium (RPMI 1640 with 10% dialyzed FCS). The final DMSO concentration never exceeded 0.5%. Vehicle controls confirmed that there was no effect of DMSO at this concentration. A water-soluble form of the disodium salt of LY231514 was used in some investigations. The recombinant enzymes used were all obtained in purified form from the following sources: rhTS from Dr. D. V. Santi (University of California at San Francisco, San Francisco, CA; Ref. 16); trifunctional mGARFT from Dr. R. G. Moran (Medical College of Virginia, Richmond, VA; Ref. 17); rhDHFR from Dr. M. Ratnam of Medical College of Ohio, Toledo, Ohio (18) and Anatrace Co. (Maumee, OH). Two forms of rhC1-S were obtained from Dr. R. E. Mackenzie (McGill University, Montreal, Quebec, Canada; Ref. 19): (*a*) the M_r 101,000 full-length enzyme of C1-S containing 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase and 10-formyltetrahydrofolate synthetase (EC 6.3.4.3) activities; and (*b*) the protein domain of C1-S containing the 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) and 5,10-methenyltetrahydrofolate cyclohydrolase activities (the M_r 35,000 truncated version of C1-S, which contained only the dehydrogenase and cyclohydrolase activities). Human AICARFT was purified as described by Rayl *et al.* (20). 10-Formyl-[6R,S]-5,6,7,8-tetrahydrofolate was prepared by a method similar to that of Rowe (21). 10-Formyl-5,8-dideazafolic acid and α,β-glycinamide ribonucleotide were prepared as described previously (2). 6R-MTHF for use in the TS assay was obtained from Eprova AG (Schaffhausen, Switzerland); the trihydrochloride salt of [6R,S]-5,6,7,8-tetrahydrofolate and the magnesium salt of [6R,S]-5,10-methylene-5,6,7,8-tetrahydrofolate for use in the C1-S dehydrogenase assay were obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). 5-Aminoimidazole-4-carboxamide ribonucleotide, AICA, folic acid, folinic acid, 7,8-dihydrofolate, NADPH, hypoxanthine, methotrexate, MTT, and thymidine were purchased from Sigma Chemical Company (St. Louis, MO). Dialyzed fetal bovine serum was purchased from Hyclone (Logan, UT). Regular and folate-free RPMI 1640 with 25 mM HEPES buffer were purchased from Whittaker Bioproducts (Walkersville, MD). The ENZFITTER microcomputer package was obtained from Biosoft (Ferguson, MO). CCRF-CEM cells were obtained from St. Jude Children's Research Hospital (Memphis, TN). HCT-8 cells were purchased from the American Type Culture Collection (Rockville, MD). CR15, a 5,10-dideazatetrahydrofolic acid-resistant CCRF-CEM subline, was described by Pizzorno *et al.* (22). ZR-75-1 human breast carcinoma cell sublines with differing folate transport properties were generously provided by Dr. K. Cowan (NCI, Bethesda, MD; Ref. 23). The GC3/C1 cell line was developed by Dr. J. Houghton (St. Jude Children's Research Hospital, Memphis, TN; Ref. 24). CCRF-CEM, HCT-8, CR15, ZR-75-1, MTX^RZR-75-1, and GC3/C1 cells were routinely cultured in RPMI 1640 medium containing L-glutamine and 25 mM HEPES buffer and supplemented with 10% dialyzed FCS. ZR-75-1 cells expressing FBP-α (MTX^RBB3-FR+ and 2FR+AA6) were cultured in folic acid-free RPMI 1640 containing L-glutamine, 25 mM HEPES buffer, 2 nM folinic acid, and 10% dialyzed FCS.

Enzyme Assays and Methods. TS activity was assayed using a spectrophotometric method described by Greene *et al.* (25), which involved monitoring the increase in absorbance at 340 nm resulting from formation of the product, 7,8-dihydrofolate. The assay buffer contained 50 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA, and 75 mM 2-mercaptoethanol, pH 7.4. The concentrations of deoxyuridylate monophosphate, 6R-MTHF, and hTS were 100 μM, 30 μM, and 30 nM (1.7 milliunits/ml), respectively. (One milliunit of enzyme activity is defined as 1 nmol of product produced per min.) At the 6R-MTHF concentration, an uninhibited reaction and six concentrations of inhibitor were assayed. K_i apparent ($K_{i\,app}$) values were determined by fitting the data to the Morrison equation (26) using nonlinear regression analysis with the aid of the program ENZFITTER. K_i values were calculated using the equation: $K_{i\,app} = K_i (1 + [S]/K_m)$, where $[S]$ is equal to 30 μM and K_m is equal to 3 μM.

DHFR activity was assayed spectrophotometrically by monitoring the disappearance of the substrates NADPH and 7,8-dihydrofolate (combined $\epsilon = 12 \times 10^3\ M^{-1}\ cm^{-1}$) at 340 nm. The reaction took place at 25°C in 0.5 ml of 50 mM potassium phosphate buffer, which contained 150 mM KCl and 10 mM 2-mercaptoethanol, pH 7.5, and 14 nM (0.34 milliunit/ml) DHFR. The NADPH concentration was 10 μM, and 7,8-dihydrofolate was varied at 5, 10, or 15 μM. At each 7,8-dihydrofolate concentration, an uninhibited reaction and seven concentrations of inhibitor were assayed. The ENZFITTER microcomputer program was used to obtain $K_{i\,app}$ values by fitting the data to the Morrison equation by nonlinear regression analysis. K_i values were calculated using the equation: $K_{i\,app} = K_i (1 + [S]/K_m)$, where $[S]$ is equal to the concentration of 7,8-dihydrofolate used and K_m of 7,8-dihydrofolate is 0.15 μM (27, 28).

GARFT activity was assayed spectrophotometrically as described previously (2) by monitoring the increase of absorbance resulting from formation of the product 5,8-dideazafolate at 295 nm. The reaction solvent contained 75 mM HEPES, 20% glycerol, and 50 mM α-thioglygerol, pH 7.5, at 25°C. The concentrations of substrates and enzyme used were 10 μM α,β-glycinamide ribonucleotide, 0–10 μM 10-formyl-5,8-dideazafolic acid, and 10 nM (1.9 milliunits/ml) GARFT. K_i values were calculated using the Enzyme Mechanism program of the Beckman DU640 spectrophotometer, which uses nonlinear regression analysis to fit data to the Michaelis-Menten equation for competitive inhibition.

AICARFT inhibition assays were carried out at room temperature by monitoring the formation of [6S]-5,6,7,8-tetrahydrofolate from 10-formyl-[6R,S]-5,6,7,8-tetrahydrofolate at A_{298}. All solutions were purged with N_2 gas prior to use. The reaction solution contained 33 mM Tris-Cl, pH 7.4, 25 mM KCl, 5 mM 2-mercaptoethanol, 0.05 mM AICA ribonucleotide, and 16 nM (2.0 milliunits/ml) of AICARFT. 10-Formyl-[6R,S]-5,6,7,8-tetrahydrofolate concentrations of 0.037, 0.074, and 0.145 mM were used (0.61, 1.23, and 2.45 times its K_m value, respectively). LY231514 was tested as an inhibitor at 0.080–0.800 mM (four concentrations). When the tri- and pentaglutamates of LY231514 were used as inhibitors, the concentrations were 0.0005–0.009 mM (eight concentrations). Enzyme assays were initiated by the addition of enzyme. Data was analyzed using the ENZFITTER program for competitive inhibition.

The method of Tan *et al.* (29) was used for assaying the activities of C1-S. This involves quenching the reaction by acid and subsequent spectrophotometric quantitation of the amount of 5,10-methenyltetrahydrofolate produced at 350 nm. Accordingly, the dehydrogenase assay was conducted in a pH 7.3 reaction mixture containing 0.084 M potassium phosphate, 0.12 M 2-mercaptoethanol, 0.17 mM NADP, 5.75–168 μM [6R,S]-5,10-methylene-5,6,7,8-tetrahydrofolate, and 0.96 nM (0.7 milliunit/ml) protein domain of C1-S containing the 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) and 5,10-methenyltetrahydrofolate cyclohydrolase activities. The synthetase assay was conducted in a pH 8.0 reaction mixture containing 0.1 M triethanolamine, 0.14 M 2-mercaptoethanol, 0.05 M KCl, 0.04 M sodium formate, 1.0 mM $MgCl_2$, 1.0 mM ATP, 62.5–2000 μM [6R,S]-5,6,7,8-tetrahydrofolate, and 1.4 nM (0.7 milliunit/ml) full-length enzyme of C1-S containing 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase and 10-formyltetrahydrofolate synthetase (EC 6.3.4.3) activities. The concentrations of LY231514 and its polyglutamates studied in each activity were from about $0.5 \times K_i$ to $3 \times K_i$. All reactions were conducted at ambient temperature (23°C) in a final volume of 0.475 ml and quenched with 0.025 ml of 0.4 M HCl. Activity data collected with a range of substrate and drug concentrations were fit to the Michaelis-Menten equation for competitive inhibition by nonlinear regression with the aid of the GRAFIT computer program (30).

***In Vitro* Cell Culture Studies.** Dose-response curves were generated to determine the concentration required for 50% inhibition of growth (IC_{50}). Test compounds were dissolved initially in DMSO at a concentration of 4 mg/ml and further diluted with cell culture medium to the desired concentration. CCRF-CEM leukemia cells in complete medium were added to 24-well Cluster plates at a final concentration of 4.8×10^4 cells/well in a total volume

of 2.0 ml. Test compounds at various concentrations were added to duplicate wells so that the final volume of DMSO was 0.5%. The plates were incubated for 72 h at 37°C in an atmosphere of 5% CO_2 in air. At the end of the incubation, cell numbers were determined on a ZBI Coulter counter. Control wells usually contained 4×10^5 to 6×10^5 cells at the end of the incubation. For several studies, IC_{50}s were determined for each compound in the presence of either 300 μM AICA, 5 μM thymidine, 100 μM hypoxanthine, or combination of 5 μM thymidine plus 100 μM hypoxanthine.

For adherent tumor cells, we used a modification of the original MTT colorimetric assay described by Mosmann (31) to measure cell cytotoxicity. The human tumor cells were seeded at 1×10^4 cells in 100 μl of assay medium/well in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA). The assay medium contained folic acid-free RPMI 1640 supplemented with 10% FCS and either 2 nM folinic acid or 2.3 μM folic acid as the sole folate source. Well 1A was left blank (100 μl of growth medium without cells). Stock solutions of antifolates were prepared in Dulbecco's PBS at 1 mg/ml, and a series of 2-fold dilutions were subsequently made in PBS. Ten-μl aliquots of each concentration were added to triplicate wells. Plates were incubated for 72 h at 37°C in a humidified atmosphere of 5% CO_2-in-air. MTT was dissolved in PBS at 5 mg/ml, 10 μl of stock MTT solution were added to each well of an assay, and the plates were incubated at 37°C for 2 additional h. Following incubation, 100 μl of DMSO were added to each well. After thorough formazan solubilization, the plates were read on a Dynatech MR600 reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The IC_{50} was determined as the concentration of drug required to inhibit cell growth by 50% compared to an untreated controls.

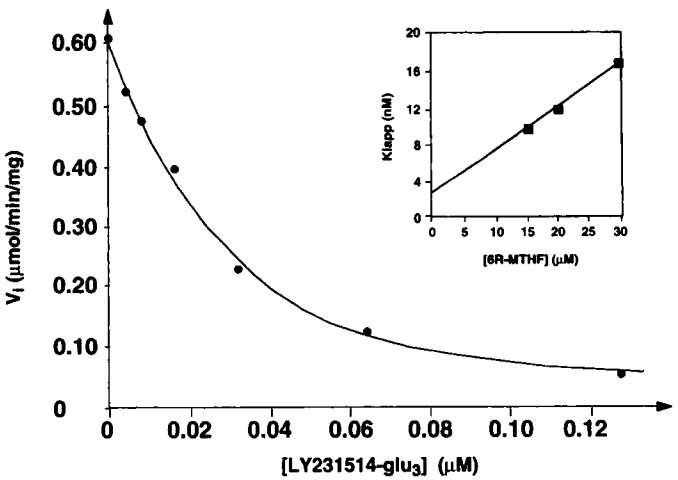

Fig. 2. Morrison Analysis of tight-binding inhibition of rhTS by LY231514-glu_3. A velocity *versus* inhibitor concentration curve is shown from a representative experiment illustrating the concentration-dependent inhibition of rhTS (29 nM) in the presence of 6R-MTHF (15 μM) and 100 μM deoxyuridylate monophosphate. *Inset*, $K_{i\ app}$ values were determined by the nonlinear fitting of data collected at three concentrations of 6R-MTHF to the Morrison equation using the ENZFITTER microcomputer package. The K_i value (1.3 nM) was determined from the slope of the graph $K_{i\ app}$ *versus* [6R-MTHF] using a K_m for 6R-MTHF of 3.0 μM.

RESULTS

Enzyme Inhibition Studies. The inhibition of rhTS by LY231514 and its polyglutamates is summarized in Table 1. The parent monoglutamate LY231514 inhibited rhTS with a K_i of 109 nM when the monoglutamated form of the substrate (6R-MTHF) (6[R]-5,10-methylenetetrahydrofolate) was used. This is in good agreement with the K_i value generated earlier for rmTS (K_i = 340 nM; Ref. 11). The longer-chain γ-glutamyl derivatives of LY231514 demonstrated significantly enhanced affinity to rhTS. The addition of two extra γ-glutamyl residues (glu_3) to LY231514 resulted in 68-fold reduction of the K_i value. Further extension of the glutamate tail (LY231514-glu_5) did not result in any significant enhancement of inhibitory potency toward rhTS. In comparison, ZD1694 was less dependent on polyglutamation. A 5-fold increase in affinity was observed for ZD1694 polyglutamates toward rhTS. It has been well recorded that mammalian TS showed a strong preference for polyglutamated folate substrates. A similar effect had been reported by Jackman *et al.* (6, 33) and Sikora *et al.* (32) in their studies of the quinazoline antifolates CB3717, ZD1694, and their polyglutamates by using partially purified L1210 murine TS. In both cases, the corresponding triglutamate derivatives demonstrated 87- and 56-fold reductions in K_i values, respectively, compared to the parent compounds. In a separate study by Cheng *et al.* (34), CB3717-glu_3 was approximately 20-fold more potent than the parent monoglutamate compound in inhibiting human TS isolated from HeLa S3 and KB cells. The quantitative differences in the reported degree of enhancement in potency as a result of polyglutamation are likely due to a combination of the variation in enzyme source used, as well as the inherent difficulty in obtaining K_i estimates for very tightly bound compounds. LY231514 and its polyglutamates inhibited rhTS in a competitive fashion with respect to the natural substrate [6R]-5,10-methylenetetrahydrofolate. The data of LY231514-glu_3 against rhTS is shown in Fig. 2. The K_i values reported in Table 1 are calculated assuming competitive inhibition for ZD1694.

Table 1 *Inhibitory activity of LY231514, ZD1694, and their polyglutamates against rhTS, rhDHFR, and rmGARFT*[a]

Compound	K_i value (nM ± SE; $n \geq 3$)		
	rhTS	rhDHFR	rmGARFT
LY231514	109 ± 9 (n = 4)	7.0 ± 1.9	9,300 ± 690
LY231514-$(glu)_3$	1.6 ± 0.1	7.1 ± 1.6	380 ± 92
LY231514-$(glu)_5$	1.3 ± 0.3	7.2 ± 0.4	65 ± 16
ZD1694	6.0 ± 0.9	45 ± 3	424,000 (336,000, 513,000)
ZD1694-$(glu)_3$	1.1 ± 0.3	37 ± 7	104,000 (81,000, 127,000)
ZD1694-$(glu)_5$	1.4 ± 0.1	30 ± 3	132,000 (124,000, 141,000)

[a] See "Materials and Methods" for assay procedures.

LY231514 was found to be a very potent inhibitor when tested against recombinant human DHFR. Tight binding analysis showed that LY231514 inhibited rhDHFR in a competitive fashion with a K_i of 7.0 nM (Table 1). In contrast to rhTS, attachment of additional γ-glutamyl residues to LY231514 had little effect on the inhibition toward rhDHFR (the glu_3 and glu_5 of LY231514 exhibited identical K_i values against rhDHFR). The polyglutamates of LY231514 also showed a competitive inhibition pattern toward rhDHFR (data not shown). It was reported that CB3717 had a K_i of 250 nM on DHFR isolated from human KB/6B cells (34) and that ZD1694 inhibited rat liver DHFR with a K_i of 93 nM (6). Likewise, polyglutamation of CB3717 and ZD1694 did not enhance affinity to DHFR. In our hands, ZD1694 and its polyglutamates also inhibited rhDHFR but were 7-fold less potent than LY231514. The polyglutamates of ZD1694 showed slight enhancement of affinity toward rhDHFR.

We also studied drug inhibition against the folate-requiring enzymes along the purine *de novo* biosynthetic pathway. LY231514 only moderately inhibited rmGARFT (K_i = 9.3 μM). Through earlier studies of 5,10-dideazatetrahydrofolates, it was discovered that GARFT inhibition is highly dependent upon the polyglutamation status of inhibitors (2). The triglutamate and pentaglutamate of LY231514 had significantly enhanced inhibitory activity against GARFT, with K_i values of 380 nM (24-fold) and 65 nM (144-fold), respectively. This makes the pentaglutamate of LY231514 a potentially potent inhibitor of purine *de novo* biosynthesis. In comparison, ZD1694 and its polyglutamates showed extremely weak inhibitory activity against GARFT. The K_i values of ZD1694, ZD1694-glu_3, and ZD1694-glu_5 were 424, 104, and 132 μM, respectively (Table 1). This result demonstrates that polyglutamyl derivatives of LY231514 are 300-2000-fold more effective than ZD1694 in inhibiting GARFT, an

Table 2 *Inhibitory activity of LY231514 and its polyglutamates against hAICARFT and the dehydrogenase and synthetase activities in C1-S*[a]

Compound	K_i value (μM)		
	5,10-methylenetetrahydrofolate dehydrogenase of C1 synthase	10-formyltetrahydrofolate synthetase of C1 synthase	AICARFT
LY231514	9.5 ± 0.9[b]	364	3.58
LY231514-$(glu)_3$	3.7	25	0.48
LY231514-$(glu)_5$	5.0	1.6	0.26

[a] See "Materials and Methods" for procedures.
[b] ± SD; n = 3.

important enzyme along the purine *de novo* biosynthetic pathway. The second folate-requiring enzyme along the purine *de novo* biosynthetic pathway is AICARFT, which uses the same folate cofactor as GARFT, 10-formyl-tetrahydrofolate, as the one carbon donor in purine biosynthesis. A similar trend of enhancement of affinity was observed for LY231514 and its polyglutamates toward hAICARFT. The K_i values observed were 3.58 μM, 480 nM (7.5-fold), and 265 nM (13.5-fold) for the mono-, tri-, and pentaglutamyl derivatives of LY231514, respectively (Table 2).

Finally, LY231514 and its polyglutamates were also found to be competitive inhibitors against both the 5,10-methylenetetrahydrofolate dehydrogenase and 10-formyltetrahydrofolate synthetase activities of C1-S (Table 2). The K_i values for the mono-, tri-, and pentaglutamyl derivatives of LY231514 were 9.5, 3.7, and 5.0 μM, respectively, for dehydrogenase and 364, 25, and 1.6 μM for synthetase. This demonstrates that the effect of polyglutamation of LY231514 on inhibition of dehydrogenase activity is marginal, but is quite significant for inhibition of synthetase activity. This observation is consistent with previous reports on the sensitivity of these two enzymes to polyglutamation status of their respective folate cofactors (35, 36). Based on the K_i values of LY231514 and its polyglutamates, the importance of C1-S as a potential target will be dependent upon the intracellular concentration of drug achieved (see below).

Cell Culture End Products Reversal Studies. Previous studies demonstrated that the antiproliferative activity of LY231514 was prevented by leucovorin but incompletely reversed by thymidine (10, 11). This suggested that aside from TS, additional enzymatic targets for this antifolate compound exist. We have now further characterized the reversal pattern of LY231514 and ZD1694 in various human tumor cell lines, including CCRF-CEM leukemia, GC3/C1 colon carcinoma, and HCT-8 ileocecal carcinoma. It was observed that 5 μM thymidine fully protected these cells from cytotoxicity with ZD1694 (Table 3). In sharp contrast, similar treatment with thymidine (5 μM) only increased the IC_{50} of LY231514 *versus* CCRF-CEM cells by 5.5-fold, GC3/C1 by 18.7-fold, and HCT-8 by 15-fold. It is interesting to note that thymidine alone produced its greatest protective effect at or near the IC_{50} of LY231514 (Fig. 3). In contrast, higher drug concentrations of LY231514 required the combination of both thymidine (5 μM) plus hypoxanthine (100 μM) to protect CCRF-CEM cells. Moreover, the combination of thymidine plus hypoxanthine totally reversed the cytotoxicity exerted by LY231514 in all three cell lines (IC_{50} values > 40 μM; Table 3). Hypoxanthine (100 μM) or aminoimidazole carboxamide (300 μM) alone did not markedly influence cytotoxicity by LY231514 (except for HCT-8 cells, in which a 5-fold

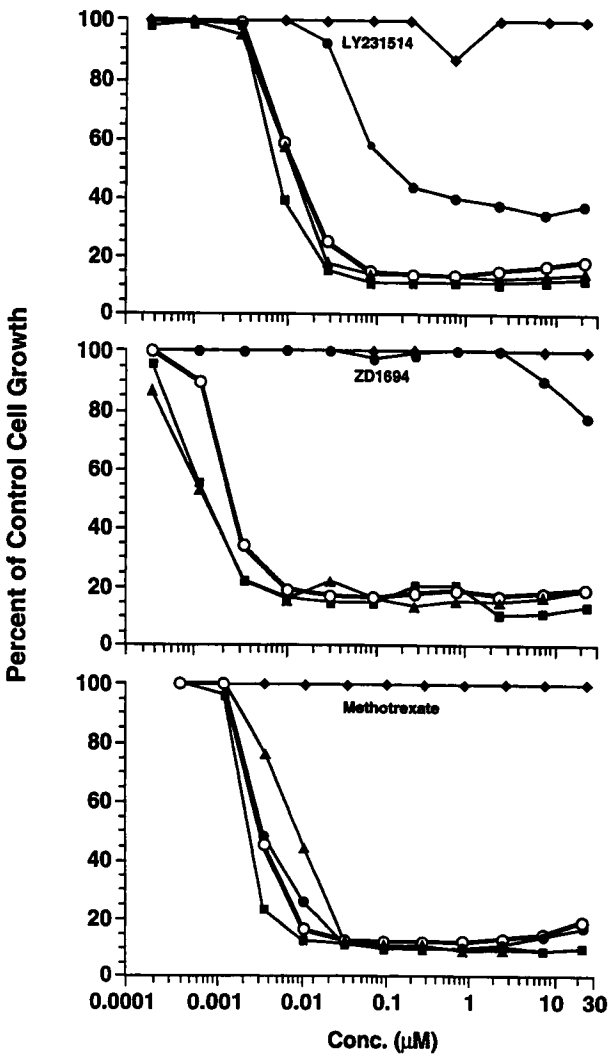

Fig. 3. End product reversal studies of LY231514, ZD1694, and methotrexate in CCRF-CEM human leukemia cells. The indicated concentrations of these compounds were incubated with cells for 72 h in the presence of a source of purines and/or thymidylate: no additions (○); 5 μM thymidine (●); 100 μM hypoxanthine (▲); 300 μM AICA (■); or 5 μM thymidine plus 100 μM hypoxanthine (◆). The reversal study of LY231514 was disclosed previously (11).

Table 3 *End products reversal studies with LY231514 and ZD1694*[a]

Cell line	IC_{50} of compound (nM)			
	Alone	+5 μM dThd	+100 μM hypoxanthine	+dTHd and hypoxanthine
LY231514				
CCRF-CEM	25	138	32	>40,000
GC3/C1	34	637	34	>40,000
HCT-8	220	3104	1077	>40,000
ZD1694				
CCRF-CEM	15	>40,000	13	>40,000
GC3/C1	4	>40,000	4	>40,000
HCT-8	65	>40,000	44	>40,000

[a] Cytotoxicity determined by MTT analysis after 72 h exposure to drug.
SE of triplicate determinations did not exceed 10% of mean.

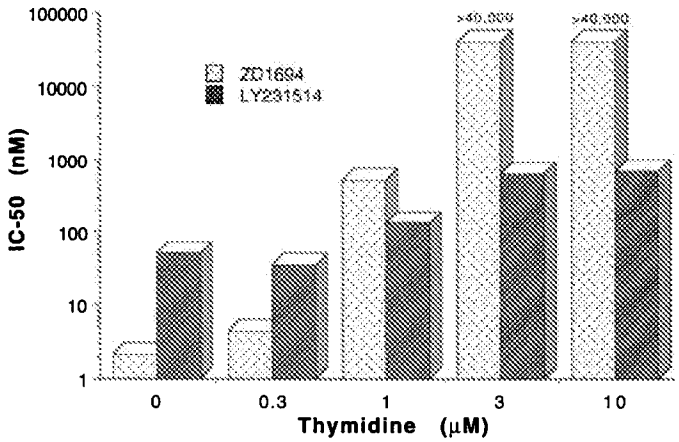

Fig. 4. Effect of thymidine on cytotoxicity of LY231514 and ZD1694 against GC3/C1 human colon carcinoma cells.

Table 4 *Antiproliferative activity of various antifolates against a 5,10-dideazatetrahydrofolate-resistant CCRF-CEM subline (CR15) and relative efficiency as substrate for FPGS*

Compound	CCRF-CEM IC_{50} (nM)[a]	CR15 IC_{50} (nM)[a,b]	FPGS (V_{max}/K_m)[c]
LY231514	25.4	>200,000	549
ZD1694	15.3	30,657	495
LY249543 (lometrexol)	9.7	>200,000	60
Methotrexate	4.2	336	4

[a] Cytotoxicity determined by MTT analysis after 72 h exposure to drug. SE of triplicate determinations did not exceed 10% of mean.

[b] CR15 cells, generously provided by Dr. G. P. Beardsley (Yale University, New Haven, CT), have markedly diminished capacity to accumulate 5,10-dideazatetrahydrofolic acid polyglutamates (their FPGS activity is approximately 10% of the wild type).

[c] Hog liver FPGS data taken from Ref. 17.

decrease in potency was observed with the addition of 100 μM of hypoxanthine). In GC3/C1 cells, the physiological concentration of thymidine in mouse plasma (1.0 μM; Ref. 24) was significantly more effective in reversing the cytotoxicity of ZD1694 than LY231514 (Fig. 4). The distinctively different reversal pattern exerted by thymidine on these two agents indicates that whereas TS may be the sole target for ZD1694, it is likely that there are other inhibitory sites for LY231514. The cell culture reversal pattern of LY231514 in CCRF-CEM cells was also distinctly different from that of methotrexate (no protection by thymidine alone; Fig. 3) and the GARFT inhibitor lometrexol (strong protection by hypoxanthine alone; data not shown; Ref. 3). These studies suggest that inhibition of DHFR and/or other enzymes along the purine *de novo* pathway may play major roles to the overall cytotoxic action of LY231514.

Role of Polyglutamation in Cytotoxicity. LY231514 has previously been demonstrated to be an exceptionally efficient substrate for FPGS (11, 12). To evaluate the role of FPGS in the cytotoxic activity of LY231514 and ZD1694, we used CR15 cells, a lometrexol-resistant CCRF-CEM subline. This subline has previously been shown to have a markedly diminished capacity to accumulate lometrexol polyglutamates and has approximately 10% of the FPGS activity of wild type cells (22). Impaired polyglutamation in CR15 cells was identified as the mechanism of resistance to lometrexol, and the cells have normal reduced folate transport activity and normal levels of the target enzyme, GARFT. We observed that CR15 cells were markedly cross-resistant to both LY231514 and ZD1694 (Table 4), suggesting that polyglutamation is a major determinant of cytotoxicity for both compounds. CR15 exhibited 7874-fold resistance to LY231514 and 2003-fold resistance to ZD1694. In comparison, methotrexate exhibited significantly less resistance in this polyglutamation-impaired subline compared to parent CEM cells.

Transport Mechanisms for Cytotoxic Activity. The roles of the RFC and FBP-α in the cytotoxic activity of LY231514 and ZD1694 were determined by using ZR-75-1 human breast carcinoma sublines that differ in expression of RFC and FBP-α (23). Wild-type ZR-75-1 cells express RFC as the major transport route for natural reduced folate cofactors and antifolate compounds and do not express detectable levels of FBP-α. The predominant role of RFC in transport of these compounds is illustrated by the fact that wild-type ZR-75-1 cells with or without transfected FBP-α were much more sensitive to drug cytotoxicity than sublines resistant to methotrexate through decreased RFC expression (Table 5). These results indicated that both LY231514 and ZD1694 are less dependent on FBP-α as the major route for internalization.

DISCUSSION

The antiproliferative activity of the "classical" antifolates depends not only on their ability to interact with intracellular folate-requiring enzyme target(s), but also on their cellular transport properties and their degree of polyglutamation. Polyglutamation, in particular, plays an essential role in determining the overall biochemical and pharmacological profiles of any given antifolate (37). The formation of polyglutamated metabolites of folates and antifolates results in the intracellular accumulation of polyglutamated metabolites to levels that are significantly higher than could otherwise be achieved at steady state by the parent compounds, and thus serves as an important cellular retention mechanism for folates and antifolates (38, 39). In addition, the resulting polyglutamates often demonstrate orders of magnitude of increased affinity toward certain target enzyme(s) (6, 32, 40). Polyglutamation may also lead to increased inhibition of other folate-dependent enzyme(s) for which the parent compounds had little or no apparent affinity.

A well-documented example of a classical antifolate is methotrexate, which was first identified as an extremely potent inhibitor of DHFR. Chabner *et al.* (41) demonstrated that methotrexate polyglutamates exhibit potent inhibition of both TS (42) and AICARFT (43), whereas the parent compound, methotrexate, had much less activity. Given the fact that methotrexate polyglutamates can accumulate in drug-sensitive cells to significantly high concentrations (estimated to be in the range of 1–10 μM in drug-sensitive cells; Refs. 38, 41, and 44–46), it is reasonable to assume that the polyglutamates of methotrexate can effectively inhibit several key enzyme systems (DHFR, TS, and AICARFT). Effective polyglutamation of methotrexate and accumulation of intracellular polyglutamates have transformed methotrexate into an agent that inhibited multiple enzymes of folate metabolism. It has been suggested that the increased activity of methotrexate polyglutamates toward other distal folate targets may be an important determinant both for its sensitivity and for selectivity in normal *versus* malignant tissues.

LY231514 is a novel pyrrolo[2,3-d]pyrimidine-based antifolate. Previous studies have demonstrated that LY231514 is one of the best

Table 5 *Antiproliferative activity of LY231514 and ZD1694 against ZR-75-1 human breast carcinoma cell lines with differing transport characteristics*[a]

Cell line	Transport	IC_{50} (nM) LY231514	IC_{50} (nM) ZD1694
Wild type	RFC+, FBP−	110.2	27.5
Wild type AA6-FR+	RFC+, FBP+	22.7	9.6
MTX^R	RFC−, FBP−	429.9	1763.2
MTX^R-BB3-FR+	RFC−, FBP+	1190.6	>20,000

[a] Cytotoxicity determined after 72 h drug exposure by MTT assay. Assay medium contained 2 nM folinic acid as the sole folate source. SE of triplicate determinations did not exceed 10% of mean. The ZR-75-1 sublines were generously provided by Dr. K. Cowan (National Cancer Institute, Bethesda, MD).

substrates that is known for the enzyme FPGS (12). *In vitro* incubation (8–24 h) of LY231514 with hog liver FPGS effectively converted LY231514 to its longer-chain polyglutamates (glu_4 and glu_5; data not shown). Whereas the parent compound LY231514 demonstrated only a moderate level of inhibition (K_i = 340 nM) against TS, the pentaglutamate of LY231514 was 100-fold more potent (K_i = 3.4 nM) in inhibiting the rmTS and correlated better with its antiproliferative activity (IC_{50} = 16 nM for CCRF-CEM cells) observed in whole cell assays (11). These data suggest that LY231514 is behaving very much like a classical antifolate, which depends highly on active membrane transport and polyglutamation as part of the activation and retention mechanism for achieving its therapeutic effects. It is thus reasonable to assume that LY231514 is a prodrug and that the polyglutamated metabolites are the responsible active species inside cells.

Two biochemically distinct transport systems, the high-affinity FBP-α and the lower-affinity RFC, have been implicated in tumor cell membrane transport of folates and antifolates (47–49). Because membrane transport is the first limiting step in the chemotherapeutic efficacy of folate analogues, and different expression levels of RFC and FBP-α are being recognized in normal and neoplastic tissues, a thorough knowledge of the relative role of each of these transport systems in the antitumor efficacy of candidate antifolates may be useful for the clinical development of novel antifolates. Westerhof *et al.* (50) have demonstrated that LY231514 and ZD1694 were efficiently transported via both transport pathways using a panel of murine L1210 leukemia cells with differing transport properties. To further investigate the mechanism(s) for LY231514 transport, we used a panel of ZR-75-1 human breast carcinoma sublines prepared by Dixon *et al.* (23) with different transport characteristics. The MTX-resistant ZR-75-1 cells that are deficient in RFC activity demonstrate 3.9-fold and 64.1-fold cross-resistance to LY231514 and ZD1694, respectively, compared to wild-type cells. Neither cell line contains detectable FBP-α. Expression of FBP-α in wild-type cells produced 4.8-fold and 2.9-fold increased sensitivity to LY231514 and ZD1694. Moreover, ZR-75-1 cells that express FBP-α but lack RFC activity (MTX^R-BB3-FR+) showed markedly decreased sensitivity toward both LY231514 and ZD1694, further suggesting a predominant role for RFC in transport of both antifolates. Differences in the involvement of FBP-α in antifolate growth-inhibitory activity between our studies and those of Westerhof *et al.* (50) may be related to the use of human *versus* murine tumor cell lines. In addition, we have recently noted that RFC is the preferential route of entry for antifolate compounds, even when mFBP-α is expressed to very high levels (51).

Pizzorno *et al.* (22) described the development and mechanisms of resistance of CCRF-CEM human lymphoblastic leukemia sublines resistant to increasing concentrations of lometrexol. The primary mechanism of resistance detected in these studies appears to be the significantly diminished accumulation of polyglutamate forms of the drug due to decreased FPGS activity. We used one of the lometrexol-resistant CCRF-CEM sublines (CR15; Ref. 22) to further test the role of polyglutamation in the growth-inhibitory activity of various antifolates, including LY231514. This line has normal levels of GARFT and normal reduced folate transport system. We observed that CR15 cells display >7874-fold cross-resistance to LY231514, 2003-fold cross-resistance to ZD1694, and 80-fold cross-resistance to MTX over a 72-h drug exposure period. The degree of resistance to methotrexate following chronic drug exposure was greater than that observed by Pizzorno *et al.* (22), and this may involve additional factors besides polyglutamation, such as levels of DHFR or intracellular pools of reduced folates. However, cross-resistance seems to correlate well with relative efficiency as substrates for FPGS for these antifolate compounds. Similarly, Jackman *et al.* (52) described an L1210 murine leukemia subline resistant to ZD1694 due to diminished FPGS levels and subsequent inability to accumulate ZD1694 or MTX polyglutamates.

In addition to greater intracellular drug retention through polyglutamation, the cytotoxic activity of LY231514 polyglutamates appears to be enhanced due to increased affinity toward multiple folate-requiring enzymes. Our data now clearly demonstrate that polyglutamates of LY231514 effectively inhibited multiple folate-requiring enzymes, a phenomenon similar to what was observed for methotrexate. LY231514 polyglutamates exhibited tight binding inhibition toward rhTS and rhDHFR, with affinity in the low nanomolar range. LY231514-glu_5 also demonstrated high affinity toward GARFT (K_i = 65 nM), making it potentially an effective inhibitor of purine biosynthesis (2). The effective transport and excellent polyglutamation profiles of LY231514 suggested that significantly high levels of LY231514 polyglutamates could be achieved intracellularly. We have found that intracellular concentrations of LY231514 and its polyglutamates can reach to a level of 10–30 μM in CCRF-CEM cells when ^{14}C-labeled LY231514 was used.[3] These high intracellular drug concentrations could result in effective inhibition of multiple enzymes (TS, DHFR, and GARFT). At these high intracellular drug concentrations, other enzymes with K_i values in the micromolar range, including C1-S and AICARFT, may also be inhibited by LY231514 polyglutamates. This simultaneous inhibition of multiple folate-dependent enzymes (TS, DHFR, GARFT, C1-S, and AICARFT) would then lead to a major disturbance of intracellular reduced folate pools and result in significant decreases in pyrimidine and purine biosynthesis.

The cell culture end product reversal pattern of LY231514 was significantly different from those of ZD1694 and methotrexate (Table 3 and Fig. 3). The distinctively different reversal pattern exerted by thymidine indicated that although TS may be the sole target for ZD1694, it is likely that there are other important inhibitory sites for LY231514. The higher degree of protection by thymidine at low drug concentrations indicated that TS is a major target for LY231514. Addition of hypoxanthine together with thymidine fully reversed the cytotoxicity of LY231514, suggesting that at higher concentrations, inhibition of DHFR and/or purine *de novo* biosynthetic enzymes were responsible for other secondary cytotoxic actions of the drug. The reversal pattern of LY231514 was also significantly different from that of methotrexate (Fig. 3). Thymidine alone did not protect the cells from the cytotoxic effect of methotrexate at all drug concentrations. The affinity of methotrexate for DHFR (K_i = 5 pM) was several orders of magnitude higher than its affinity for TS (K_i = 0.047 μM for MTX-glu_5), suggesting that the primary intracellular target of methotrexate may still be DHFR and not TS.

Knowledge from *in vitro* studies of individual folate-dependent enzymes by antifolates have been incorporated into metabolic models that describe folate cycle kinetics in murine (53) and human (54, 55) systems for the purpose of evaluating multiple folate enzyme inhibition by methotrexate polyglutamates. A clear understanding of the relationship between the intracellular pools of reduced folates and LY231514 polyglutamates under various drug exposure and rescue conditions will be tremendously useful in assessing the relative significance of inhibiting each individual enzyme by LY231514 and its metabolites (41).

In summary, through enzymatic and cellular studies, we have demonstrated that as a result of polyglutamation, LY231514 can achieve high enough intracellular concentrations that it may drastically affect folate metabolism through blockade at TS, DHFR, and GARFT, and to a lesser extent at AICARFT and C1-S. The combined

[3] R. M. Schultz, unpublished observation.

effects of the inhibition exerted by LY231514 at each target gives rise to an unusual end product reversal pattern at the cellular level that is distinct from those of other inhibitors such as methotrexate and the quinazoline antifolates. This may explain the encouraging Phase I results of activity in advanced stages of colorectal and pancreatic cancer (14). A broad Phase II program is currently under way to investigate a variety of resistant solid tumors, including colorectal, breast, non-small cell lung, pancreatic, and other gastrointestinal tumors.

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Electronic Acknowledgement Receipt

EFS ID:	6448216
Application Number:	11776329
International Application Number:	
Confirmation Number:	6568
Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Customer Number:	25885
Filer:	Elizabeth Ann McGraw/Lisa Capps
Filer Authorized By:	Elizabeth Ann McGraw
Attorney Docket Number:	X14173B
Receipt Date:	13-NOV-2009
Filing Date:	11-JUL-2007
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Application Type:	Utility under 35 USC 111(a)

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Payment Type	Deposit Account
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1		X14173BRejectionResponse.pdf	180100 a59ce243c93a4578a16e5add9ac6272799a a8051	yes	12

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Claims	2	4
Applicant Arguments/Remarks Made in an Amendment	5	12

Warnings:

Information:

2	Information Disclosure Statement (IDS) Filed (SB/08)	X14173BIDSForm.pdf	609326 bf1b29b43261a56e4ab4108f910bd0f84b9 8e949	no	5

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6	NPL Documents	X14173BNO5.pdf	5142649 bcf2211f20db5008d570807632dafad05d5c 7930	no	39

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7	NPL Documents	X14173BNO6.pdf	2535413 73bea32d9ef3bb98117403bc34ae678283d92985	no	24
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Warnings:					
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11	NPL Documents	X14173BNO10.pdf	151330 ee0f7dbe3ed827210e41224b3ff1396a41a7b9a4	no	1
Warnings:					
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12	NPL Documents	X14173BNO11.pdf	450474 3f21fbd2993d3b4707f0d11226f7d24b6e9af7dd	no	3
Warnings:					
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13	NPL Documents	X14173BNO12.pdf	1807543 15ead8b759dd589d2fa479738f0ac82c320e22fd	no	8
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16	NPL Documents	X14173BNO1.pdf	217050 9ef8cb852363fbe241ff173f1d92b54819b00d15	no	23
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 11/776,329

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)		OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	x 25 =		OR	x 50 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	x 105 =			x 210 =	
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))			185			370	
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

11-13-09

AMENDMENT A	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)		OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 22	Minus	** 23	= 1	x 25 =		OR	x 50 =	
Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	=	x 105 =		OR	x 210 =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					185		OR	370	
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x 25 =		OR	x 50 =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x 105 =		OR	x 210 =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					185		OR	370 N/A	
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/776,329	07/11/2007	Clet Niyikiza	X14173B	6568

25885 7590 11/19/2009

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

EXAMINER	
WEDDINGTON, KEVIN E	

ART UNIT	PAPER NUMBER
1614	

NOTIFICATION DATE	DELIVERY MODE
11/19/2009	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patents@lilly.com

Interview Summary	Application No.	Applicant(s)	
	11/776,329	NIYIKIZA ET AL.	
	Examiner	Art Unit	
	KEVIN WEDDINGTON	1614	

All participants (applicant, applicant's representative, PTO personnel):

(1) *KEVIN WEDDINGTON*. (3)*Bill McMillen*.

(2) *Elizabeth A. McGraw*. (4)_____.

Date of Interview: *12 November 2009*.

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☒ Yes e)☐ No.
If Yes, brief description: *Proposed Amendment (Right-Faxed)*.

Claim(s) discussed: *The claims in general*.

Identification of prior art discussed: *The pior art of record*.

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☒ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *The attorney of record, Ms. McGraw, explained the proposed amendment with the response to the outstanding rejections. The attorney will officially submit the proposed amendment*.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

/KEVIN WEDDINGTON/ Primary Examiner, Art Unit 1614	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

———

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

The Oncologist®
FDA Commentary

FDA Drug Approval Summaries: Pemetrexed (Alimta®)

MAITREYEE HAZARIKA, ROBERT M. WHITE, JOHN R. JOHNSON, RICHARD PAZDUR

Division of Oncology Drug Products, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Rockville, Maryland, USA

Key Words. *Pemetrexed · Alimta® · Malignant pleural mesothelioma*

LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Explain the mechanism of action of a recently approved new cancer drug, pemetrexed.
2. Describe the adverse-event profile of pemetrexed and a novel approach for toxicity reduction.
3. Discuss the rationale for the FDA approval of pemetrexed.

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ABSTRACT

The purpose of this report is to summarize information on pemetrexed (LY231514; MTA; Alimta®; Eli Lilly and Company; Indianapolis, IN), a drug recently approved by the U.S. Food and Drug Administration (FDA). The review of the efficacy and safety of pemetrexed is summarized below. Pemetrexed is a pyrrolopyrimidine antifolate. It inhibits thymidylate synthase, glycinamide ribonucleotide formyltransferase, and dihydrofolate reductase. In a single, randomized, single-blind, multicenter phase III trial, the efficacy and safety of pemetrexed combined with cisplatin (Platinol®; Bristol-Myers Squibb; Princeton, NJ) were compared with those of single-agent cisplatin in 448 patients with malignant pleural mesothelioma. Two hundred twenty-six patients were randomized to receive pemetrexed and cisplatin, while 222 patients were randomized to receive cisplatin alone. The primary study end point was survival. Median survival times were 12.1 months for the pemetrexed plus cisplatin treated arm and 9.3 months for the cisplatin alone arm. Pemetrexed causes myelosuppression. The most common adverse events were neutropenia, fatigue, leukopenia, nausea, dyspnea, and vomiting.

On February 4, 2004, pemetrexed was approved by the FDA in combination with cisplatin for the treatment of patients with malignant pleural mesothelioma whose disease is unresectable or who are otherwise not candidates for curative surgery. The recommended dose of pemetrexed is 500 mg/m² administered as an i.v. infusion over 10 minutes on day 1 of each 21-day cycle together with cisplatin at a dose of 75 mg/m² infused over 2 hours beginning 30 minutes after the pemetrexed infusion. Patients must receive oral folic acid and vitamin B_{12} injections prior to the start of therapy and continue these during therapy to reduce severe toxicities. Patients should also receive corticosteroids with chemotherapy to reduce the risk of skin rashes. Approval was based on superior survival as a clinical benefit. *The Oncologist 2004;9:482-488*

Correspondence: Maitreyee Hazarika, M.D., U.S. Food and Drug Administration, HFD-150, 5600 Fishers Lane, Rockville, Maryland 20857, USA. Telephone: 301-594-2473; Fax: 301-594-0499; e-mail: HazarikaM@cder.fda.gov *Received June 8, 2004; accepted for publication July 20, 2004.* *1083-7159/2004/$12.00/0*

Introduction

Malignant mesotheliomas arise primarily from the surface serosal cells of the pleural, peritoneal, and pericardial cavities and are highly aggressive neoplasms. The etiology of malignant mesothelioma is primarily exposure to asbestos fibers [1]. Simian virus 40 has also been implicated in the etiology [2]. Malignant pleural mesothelioma (MPM) most typically develops 20-50 or more years after the first documented asbestos exposure, commonly in the fifth to seventh decade of life. With median survival durations of 10-17 months from onset of symptoms and 9-13 months from diagnosis, the prognosis is poor for patients with these neoplasms.

Although the Cancer Committee of the College of American Pathologists provides a protocol for the examination of specimens from patients with MPMs, histological diagnosis remains difficult [3]. Earlier staging systems were not uniform, and the International Mesothelioma Interest Group Staging System (IMIG) updated several earlier staging systems after taking into consideration information about the impact of tumor (T) and nodal (N) status on survival [4]. Evaluations with two series of patients validated the staging system [5, 6].

Surgical resection of MPM is possible in only a minority of patients. Fewer than 15% of these patients live beyond 5 years [6, 7]. Curative radiotherapy, although available, is limited by the tumor volume to be treated and by toxicities to surrounding normal tissue [8]. Chemotherapy with single agents, such as doxorubicin (Adriamycin®; Bedford Laboratories; Bedford, OH), methotrexate with rescue, 5-azacytadine, 5-fluorouracil, cisplatin (Platinol®; Bristol-Myers Squibb; Princeton, NJ), and gemcitabine (Gemzar®; Eli Lilly; Indianapolis, IN), appears to have limited activity [9-11]. Combination chemotherapy regimens have shown response rates that range from 0%-48%, with the highest reported for cisplatin and gemcitabine (48% in 21 patients) [12, 13]. None of the single or combination chemotherapy regimens provide a survival benefit.

A New Drug Application for a first-line indication for pemetrexed (LY231514; MTA; Alimta®; Eli Lilly and Company; Indianapolis, IN) for patients with MPM not candidates for surgical resection was submitted to the U.S. Food and Drug Administration (FDA) in October 2002. At the time of the submission, there were no FDA-approved drugs for MPM. Response rate was originally proposed by the applicant as the primary end point for the randomized study, as they believed that unidimensional measurements were sufficient to provide information on response. Due to uncertainty about the application of unidimensional measurements of pleural rind for response assessments and uncertainty regarding the relationship of response to clinical benefit, the FDA required survival as the primary end point.

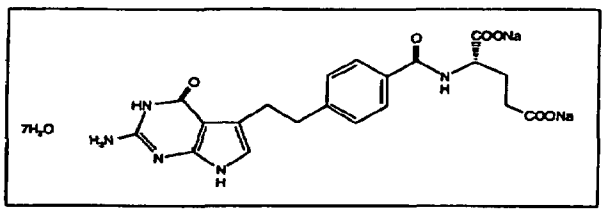

Figure 1. Chemical structure of pemetrexed (pemetrexed sodium).

Background

Pemetrexed (pemetrexed disodium) (L-glutamic acid, N-[4-[2-(2-amino-4,7-dihydro-4-oxo-1H-pyrrolo[2,3-d] pyrimidin-5-yl) ethyl] benzoyl]-, disodium salt) is a structurally novel antifolate that possesses the unique 6-5 fused pyrrolo [2,3-d] pyrimidine nucleus (Fig. 1). It is transported into cells by both the reduced folate carrier and membrane folate-binding protein transport systems. It was found to be one of the best substrates for the folylpoly-gamma-glutamate synthetase [14]. Polyglutamation and polyglutamated metabolites of pemetrexed are believed to have a role in the selectivity and antitumor activity of this agent. The polyglutamated forms have a greater affinity for thymidylate synthase and glycinamide ribonucleotide formyltransferase (GARFT) than pemetrexed monoglutamate. They inhibit thymidylate synthase, GARFT, and dihydrofolate reductase, all folate-dependent enzymes involved in the de novo biosynthesis of thymidine and purine nucleotides (Fig. 2).

Pemetrexed is primarily eliminated in the urine, with 70%-90% of the dose recovered as unchanged parent drug within the first 24 hours. It is not metabolized to any appreciable extent. It does not inhibit the cytochrome P-450 (CYP) isoenzymes CYP3A4, CYP2D6, CYP1A2, or CYP2C9. The terminal half-life from plasma is 3.5 hours in patients with normal renal function. It is approximately 81% bound to plasma proteins. Total plasma clearance of pemetrexed

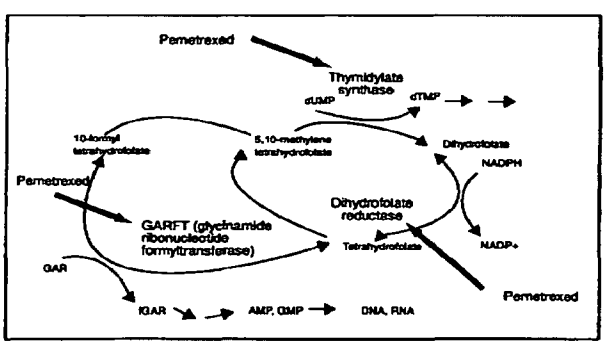

Figure 2. Inhibition of multiple folate-requiring enzymes by pemetrexed and its polyglutamates.

decreases as renal function decreases. There have been no pharmacokinetic evaluations in patients with third-space accumulations.

PHASE III STUDY

Patients with histologically confirmed MPMs who were not candidates for curative surgery were enrolled and were randomized equally between the two treatment arms of pemetrexed plus cisplatin and cisplatin alone. Clinical staging was done using the IMIG staging criteria. Patients were entered and randomly assigned to a treatment arm based on local pathology; independent centralized pathology review was carried out on patients only when feasible. Eligibility criteria required the presence of unidimensionally and/or bidimensionally measurable disease. Patients were excluded if they had received prior systemic chemotherapy, intracavitary cytotoxic drugs, or immunomodulators. The primary end point was survival. The results of the phase III trial were previously reported [15].

Between May 1999 and November 2001, 456 patients were considered eligible for the trial, and they constituted the intent-to-treat population. Of these, 448 patients were treated and were considered assessable for efficacy and toxicity analyses: 226 patients in the pemetrexed plus cisplatin arm and 222 patients in the cisplatin alone arm. The primary safety analysis was performed on a subset of patients who had received supplementation with folic acid and vitamin B_{12} injections: 168 patients on the pemetrexed plus cisplatin arm and 163 patients on the cisplatin alone arm. The patients were predominantly male, Caucasian, with good performance status scores (Table 1). Median age was 61 years (range 19-85 years). In the pathologically confirmed mesothelioma patients, 85% had epithelial histologies. In confirmed mesothelioma pathology patients, 78% had stage III or stage IV disease. In the randomized and treated population, 7.5% of patients had received prior chemotherapy for the purpose of pleurodesis, while 9.7% had received prior radiotherapy that had concluded at least 4 weeks before enrollment.

In the pemetrexed plus cisplatin treatment arm, pemetrexed was administered at a dose of 500 mg/m² diluted in approximately 100 ml of normal saline as a 10-minute i.v.

Table 1. Summary of patient characteristics

Patient characteristic	Randomized and treated patients		Fully supplemented patients	
	Pemetrexed + cisplatin (n = 226)	Cisplatin (n = 222)	Pemetrexed + cisplatin (n = 168)	Cisplatin (n = 163)
Age (years)				
Median (range)	61 (29-85)	60 (19-84)	60 (29-85)	60 (19-82)
Gender (%)				
Male	184 (81.4)	181 (81.5)	136 (81.0)	134 (82.2)
Female	42 (18.6)	41 (18.5)	32 (19.0)	29 (17.8)
Origin (%)				
Caucasian	204 (90.3)	206 (92.8)	150 (89.3)	153 (93.9)
Hispanic	11 (4.9)	12 (5.4)	10 (6.0)	7 (4.3)
Asian	10 (4.4)	4 (1.9)	7 (4.2)	3 (1.8)
African descent	1 (0.4)	0	1 (0.6)	0
Stage at entry (%)				
I	16 (7.1)	14 (6.3)	15 (8.9)	12 (7.4)
II	35 (15.6)	33 (15.0)	27 (16.2)	27 (16.8)
III	73 (32.4)	68 (30.6)	51 (30.5)	49 (30.4)
IV	101 (44.9)	105 (47.2)	74 (44.3)	73 (45.3)
Unspecified	1 (0.4)	2 (0.9)	1 (0.6)	2 (1.2)
Diagnosis/histology[a] (%)				
Epithelial	154 (68.1)	152 (68.5)	117 (69.6)	113 (69.3)
Mixed	37 (16.4)	36 (16.2)	25 (14.9)	25 (15.3)
Sarcomatoid	18 (8.0)	25 (11.3)	14 (8.3)	17 (10.4)
Other	17 (7.5)	9 (4.1)	12 (7.1)	8 (4.9)
Baseline KPS score[b] (%)				
70-80	109 (48.2)	97 (43.7)	83 (49.4)	69 (42.3)
90-100	117 (51.8)	125 (56.3)	85 (50.6)	94 (57.7)

[a]Only 67% of the patients had the histologic diagnosis of malignant mesothelioma confirmed by independent review.

[b]Karnofsky performance scale.

infusion followed by the administration of cisplatin 30 minutes later at a dose of 75 mg/m^2 over 2 hours. In the cisplatin alone treatment arm, approximately 100 ml of normal saline were given as an i.v. infusion over approximately 10 minutes followed 30 minutes later by the same dose of cisplatin as used in the alternate treatment arm.

Folic acid and vitamin B_{12} supplements were introduced into the trial for safety reasons. The sponsor initiated a multivariate analysis in 1997 to assess the relationships between vitamin metabolites, drug exposure, and other baseline patient characteristics and toxicity following therapy with pemetrexed [16]. It was concluded that toxicity from pemetrexed therapy appeared to be higher in patients with elevated pretherapy homocysteine levels and that elevated baseline homocysteine levels correlated with severe toxicities, such as febrile neutropenia, grade 4 neutropenia, thrombocytopenia, and diarrhea. As a result, vitamin supplementation was given to patients in both treatment arms to preserve blinding. Folic acid (350-1,000 μg daily) was given orally, daily starting 7 days before the first dose of chemotherapy and was continued while the patient was on therapy and for 21 days after cessation of therapy. Vitamin B_{12} injections (1,000 μg i.m.) were started 1 week before the first dose of chemotherapy and were repeated every 3 cycles while the patient was on therapy.

Patients were also given dexamethasone the day before, the day of, and the day after chemotherapy administration to reduce the risk of skin rashes. Dexamethasone was given to all patients in both arms.

Randomized Clinical Trial Results

Methods

The pemetrexed marketing application was based on a single, randomized, single-blind, phase III, multicenter study that included 88 principal investigators who enrolled 574 patients in 20 countries.

Although the intent-to-treat population numbered 456 patients, the applicant's efficacy claim was based on the 448 patients in the randomized and treated population. The primary analysis was a comparison of survival times between the two treatment arms in the randomized and treated group. Differences were assessed using a two-sided log-rank test. A planned interim analysis was conducted and presented to the Data Safety Monitoring Board. Because of this interim analysis, the final comparison of survival was tested at the $\alpha = 0.0476$ level.

Survival

In the 448 randomized and treated patients, the survival time for patients treated with pemetrexed plus cisplatin was longer than the survival time for patients treated with cisplatin alone—median 12.1 months versus 9.3 months ($p = 0.021$, hazard ration [HR] = 0.77, 95% confidence interval [CI] of HR = 0.61-0.96) (Table 2). In the subgroup of the fully folic acid- and vitamin B_{12}-supplemented patients ($n = 331$), the median survival times for patients treated with pemetrexed plus cisplatin and cisplatin alone were 13.3 months and 10 months, respectively ($p = 0.051$, HR = 0.76, 95% CI of HR = 0.57-1.0).

Since only 67% of the randomized and treated patients had the diagnosis of mesothelioma confirmed by independent review, the FDA conducted an independent survival analysis on the pathologically confirmed mesothelioma subset. In the randomized and treated patients ($n = 303$), the median survival times for patients treated with pemetrexed plus cisplatin and cisplatin alone were 13 months and 10.2 months, respectively ($p = 0.066$). In the subgroup of the fully folic acid- and vitamin B_{12}-supplemented patients ($n = 220$) that were pathologically confirmed, the median survival times for patients treated with pemetrexed plus cisplatin and cisplatin alone were 14.4 months and 10.3 months, respectively ($p = 0.058$).

The FDA also found the intent-to-treat analysis (with the inclusion of the eight patients, i.e., $n = 456$) comparable with the randomized and treated analysis ($n = 448$) of survival. Kaplan-Meier survival curves for the randomized and treated group are shown in Figure 3.

Safety

The primary safety analysis was done on the fully vitamin-supplemented subgroup, which consisted of 168 patients on the pemetrexed plus cisplatin arm and 163 on the cisplatin alone arm.

Neutropenia (24.4%), fatigue (17.3%), leukopenia (15.5%), nausea (11.9%), dyspnea (11.3%), and vomiting (10.7%) were the most commonly reported grade 3 and 4 adverse events (Table 3). Febrile neutropenia and neutropenic sepsis were relatively infrequent. The incidences of grade 3

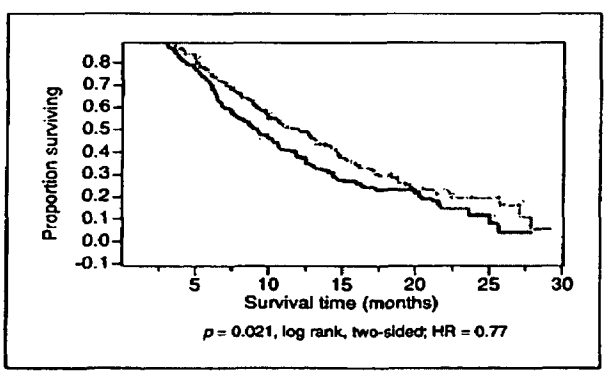

Figure 3. Kaplan-Meier survival curves for all randomized treated patients.

Table 4. Adverse events in fully supplemented patients

Adverse Events	Pemetrexed + cisplatin $n = 168$		Cisplatin $n = 163$	
	All grades (%)	Grades 3 and 4 (%)	All grades (%)	Grades 3 and 4 (%)
Laboratory				
Hematologic				
Neutropenia	57.1	24.4	13.5	3.1
Leukopenia	54.8	15.5	18.4	0.6
Anemia	33.9	6.0	14.7	0.6
Thrombocytopenia	26.2	5.4	9.2	0.0
Renal				
Creatinine	15.5	0.6	11.0	1.2
Renal Failure	2.4	0.0	1.2	0.0
Hepatic				
SGOT (AST)	8.3	0.0	6.1	0.6
SGPT (ALT)	6.0	0.0	10.4	0.6
Clinical				
Constitutional Symptoms				
Fatigue	81.5	17.3	73.6	12.9
Fever	17.3	0.0	8.6	0.0
Other constitutional symptoms	10.7	2.4	8.0	1.2
GastroIntestinal				
Nausea	84.5	11.9	78.5	5.5
Vomiting	58.9	10.7	50.9	4.3
Constipation	46.4	3.6	40.5	0.6
Anorexia	35.1	2.4	27.0	0.6
Stomatitis/pharyngitis	28.0	3.0	8.0	0.0
Diarrhea without colostomy	25.6	3.6	15.3	0.6
Dysphagia, esophagitis, odynophagia	6.0	1.2	5.5	0.0
Other gastrointestinal symptoms	19.6	1.8	16.0	0.6
Cardiovascular				
Hypertension	26.2	11.3	34.4	17.8
Edema	14.3	1.2	15.3	2.5
Thrombosis/embolism	7.1	6.0	3.7	3.7
Pulmonary				
Dyspnea	65.5	11.3	63.2	9.2
Pleuritic pain	17.3	1.8	19.0	4.9
Cough	38.1	0.6	37.4	1.2
Other pulmonary symptoms	20.2	3.0	19.0	2.5
Pain				
Tumor pain	18.5	4.8	14.7	4.3
Chest pain	40.5	8.3	30.7	6.7
Other pain	15.5	3.0	25.8	4.3
Neurology				
Neuropathy—sensory	17.3	0.0	14.7	0.6
Mood alteration—depression	13.7	1.2	9.2	1.2
Mood alteration—anxiety agitation	13.1	0.6	8.6	0.0
Infection/febrile neutropenia				
Infection without neutropenia	12.5	2.4	4.3	0.0
Infection with grade 3 or 4 neutropenia	6.0	0.6	3.7	0.0
Infection/febrile neutropenia—other	3.0	1.2	1.8	0.0
Febrile neutropenia	0.6	0.6	0.6	0.0
Dermatology/skin				
Rash/desquamation	22.0	0.6	9.8	0.0
Immune				
Allergic reaction/hypersensitivity	2.4	0.0	0.6	0.0

Abbreviations: SGOT (AST) = serum glutamic-oxaloacetic transaminase (aspartate aminotransferase): SGPT (ALT) = serum glutamic-pyruvic transaminase (alanine aminotransferase).

Table 2. Efficacy of pemetrexed plus cisplatin versus cisplatin in MPM patients

Efficacy parameter	Randomized and treated patients		Fully supplemented patients	
	Pemetrexed + cisplatin (n = 226)	Cisplatin (n = 222)	Pemetrexed + cisplatin (n = 168)	Cisplatin (n = 163)
Median overall survival	12.1 months	9.3 months	13.3 months	10.0 months
(95% CI)	(10.0, 14.4)	(7.8, 10.7)	(11.4, 14.9)	(8.4, 11.9)
Hazard ratio	0.77		0.76	
Log rank p value[a]	0.021		0.051	
Percent censored	35.8	28.4	43.5	36.8

[a]log rank, two-sided.

and 4 anemia and thrombocytopenia were 6% and 5.4%, respectively, in patients on the pemetrexed plus cisplatin arm (Table 4). The most common clinical cause of dose delay in both arms was neutropenia, followed by reduced creatinine clearance, leukopenia, anemia, stomatitis, and infection. Cycle 4 was the cycle of therapy with the most clinical delays in both treatment arms.

Toxicities were higher in the pemetrexed plus cisplatin arm than in the cisplatin alone arm. Severe toxicities were reduced with the use of folic acid and vitamin B_{12} supplementation.

Table 3. Common adverse events (>10% of patients) in fully vitamin-supplemented patients

Adverse event	Pemetrexed + cisplatin (%) n = 168	Cisplatin (%) n = 163
Decreased neutrophils	24.4	3.1
Fatigue	17.3	12.9
Leukopenia	15.5	0.6
Nausea	11.9	5.5
Dyspnea	11.3	9.2
Vomiting	10.7	4.3

Discussion

In a single, randomized, single-blind trial, the combination of pemetrexed and cisplatin, compared with cisplatin alone, showed a statistically significant longer overall survival in MPM patients. Pemetrexed plus cisplatin is the first treatment for MPM to demonstrate a survival benefit. The trial was changed while ongoing, and supplementation with folic acid and vitamin B_{12} was added.

Although a single trial, a large number of independent investigators from multiple international centers contributed data to the trial, and there was a substantial increment in survival of 3 months. The efficacy of pemetrexed was supported by an improvement in pulmonary function tests.

Survival analyses in all intent-to-treat patients and in the randomized and treated patients both favored the pemetrexed plus cisplatin group at a statistically significant level. Survival analyses in the fully vitamin-supplemented subgroup and in the subgroup with a confirmed histologic diagnosis of MPM also favored the pemetrexed plus cisplatin group at a borderline statistical significance level.

Similar to the approved label of pemetrexed, numerical values for response rate are not mentioned in the body of this article. As anticipated prior to the study, there was considerable discrepancy in tumor response evaluations among the study investigators, the study independent reviewers, and the FDA reviewers. The FDA review of the submitted images could confirm tumor response in only 47 of the 94 patients in the pemetrexed plus cisplatin treatment group for whom the applicant claimed a tumor response. Although tumor response rate appeared higher in the pemetrexed plus cisplatin treatment group, the exact numbers are very uncertain.

Following therapy with pemetrexed, toxicities appeared to be higher in patients with elevated pretherapy homocysteine levels. Elevated baseline homocysteine levels (≥10 μmol/l) highly correlated with severe hematological and nonhematological toxicities. Thus, every patient since December 1999 treated in the trial with pemetrexed was supplemented with folic acid and vitamin B_{12} to improve patient safety.

In patients treated with the combination therapy with full vitamin supplementation, the common adverse events were neutropenia, fatigue, leukopenia, nausea, vomiting, and dyspnea. In comparison with the nonsupplemented subgroup of patients, toxicities were reduced by folate and vitamin B_{12} supplementation. Despite supplementation, the combination of pemetrexed and cisplatin produces a high degree of toxicity.

For MPM, the recommended dose of pemetrexed is 500 mg/m^2 administered as an i.v. infusion over 10 minutes on day 1 of each 21-day cycle followed by cisplatin at a dose of 75 mg/m^2 infused over 2 hours beginning 30 minutes after the pemetrexed infusion. Folic acid (350-1.000 μg daily) orally, daily must be started 1-3 weeks before the first dose of

chemotherapy and continued while the patient is on therapy. Vitamin B_{12} (1,000 μg i.m.) injections must be started 1-3 weeks before the first dose of chemotherapy and repeated every 9 weeks while the patient is on therapy. Patients should also be given dexamethasone on the day before chemotherapy for a total of 3 days to reduce the risk of skin rash.

On February 4, 2004, the FDA approved pemetrexed for use in combination with cisplatin for the treatment of MPM in patients whose disease is either unresectable or who are not otherwise candidates for curative surgery. Approval was based on a longer survival time for patients treated with pemetrexed plus cisplatin than for those treated with cisplatin alone.

ACKNOWLEDGMENT

The views expressed are the result of independent work and do not necessarily represent the views and findings of the U.S. FDA.

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Alimta® (pemetrexed)

Consistent with the company's policy of public data disclosure, this clinical trial results section includes result summaries of completed Lilly-sponsored clinical studies conducted on Lilly marketed products since July 1, 2004. The results of all Phase I, II, and III trials conducted in support of a product's initial registration will be disclosed regardless of outcome, no later than when the first indication is approved and the drug is commercially available for patient use anywhere in the world. The results of all subsequent Phase II, III, and IV trials conducted after initial approval will be similarly disclosed within one year of trial completion. A trial's results, irrespective of study phase, will be disclosed as soon as possible if there are significant safety findings.

Consistent with ICH E3 guidelines, Lilly will disclose the results of primary and secondary outcome measures that are specified in the study protocol, as well as additional safety and efficacy results that impact patient care and the use of our products. Also, Lilly discloses a comprehensive description of the trial design and methodology for each study. Results will be disclosed regardless of whether they support the hypothesis being tested or are contrary to the predicted outcome. Clinical trial results will be publicly disclosed as stated above, unless posting would compromise publication in a peer reviewed medical journal or contravene national laws or regulations. Results of studies that are under review by peer-reviewed journals that prohibit pre-publication disclosure will be posted on the registry at the time of publication. Clinical trial results are also disclosed through presentations and abstract submissions at professional scientific meetings.

The registry is also populated with the results of core efficacy and safety registration trials for products first approved after July 1, 1994.

Included are trial result summaries that are completed to date. The company will continue to disclose additional summaries and publication citations as they become available.

Summaries of clinical study results are intended to report the results of the study that were known at the time of each study's completion. The information contained in these summaries is not intended to promote or otherwise commercialize (directly or indirectly) any off-label or unapproved uses of Lilly medicines, nor is it intended to provide a comprehensive analysis of all data currently available regarding a particular drug. In some cases, more current information regarding a drug, including post-hoc analyses and meta-analyses, may be available as part of the general body of scientific knowledge in forms such as manuscripts, abstracts, or posters.

Clinical studies on Lilly products may also be sponsored and reported by parties other than Lilly in other clinical trial registries.

Trial Results

The following trials have been conducted for Alimta.

Disease	Trial ID	Trial Title	Trial Phase	Results	Citations
Adenocarcinoma	9508	Phase II Study of Gemcitabine and Pemetrexed in Primary Unknown Adenocarcinoma	2	9508 Results	
Bladder Cancer	4698	A Phase 2 Study of ALIMTA (Pemetrexed) in Patients with Advanced or Metastatic Recurrent Transitional Cell Carcinoma of the Urothelium	2	4698 Results	4698 Citations
Bladder Cancer	4699	A Phase 2 Trial of ALIMTA® Plus Gemcitabine in Locally Advanced or Metastatic Transitional Cell Carcinoma of the Urothelium	2	4699 Results	4699 Citations
Bladder Cancer	8279	Phase II Trial of Pemetrexed Disodium and Gemcitabine in Advanced Urothelial Cancer	2	8279 Results	
Breast Cancer	4028	A Phase 1/2 Dose-Escalating Study of ALIMTA (pemetrexed) and Epirubicin Administered Every 21 Days in Patients with Locally Advanced or Metastatic Breast Cancer	1 / 2	4028 Results	
Breast Cancer	2245	A Phase II Study of a Combination of Pemetrexed and Gemcitabine in Patients with Metastatic Breast Cancer: an NCCTG Study	2	2245 Results	2245 Citations
Breast Cancer	7491	A Randomized, Double-Blind, Phase 2 Study of Two Doses of ALIMTA® as First-Line Chemotherapy for Advanced Breast Cancer	2	7491 Results	7491 Citations
Breast Cancer	7771	A Phase 2 Study of ALIMTA and Carboplatin in the Treatment of Patients with Locally Advanced or Metastatic Breast Cancer	2	7771 Results	
Breast Cancer	9305	A Phase 2 Study of Biweekly Pemetrexed and Gemcitabine in Patients with Metastatic Breast Cancer	2	9305 Results	
Colorectal Cancer	2927	A Phase 1/2 Trial of Pemetrexed Plus Irinotecan Administered Every 21 Days in Patients with Previously Treated Locally Advanced or Metastatic Colorectal Cancer	2	2927 Results	2927 Citations
Colorectal Cancer	5142	Phase II Trial Of Alimta Plus Oxaliplatin Administered Every 21 Days For First-Line Treatment Of Patients With Advanced Colorectal Cancer	2	5142 Results	5142 Citations
Gastric Cancer	6154	Open-Label Single-Arm Phase 2 Study of ALIMTA plus Cisplatin in Korean Patients with Advanced Gastric Carcinoma	2	6154 Results	6154 Citations

Gastric Cancer	8059	Pemetrexed Plus Oxaliplatin in the Management of Advanced Gastric Cancer: A Multicenter Phase II Trial	2	8059 Results	
Liver Cancer	9417	Single-Agent Pemetrexed in Patients with Advanced or Metastatic Hepatoma	2	9417 Results	
Malignant Pleural Mesothelioma	1307	Phase I trial of LY231514 and cisplatin every 21 days in patients with locally advanced or metastatic solid tumors	1	1307 Results	1307 Citations
Malignant Pleural Mesothelioma	1806	A Phase 1 Pharmacokinetic Trial of Alimta® (pemetrexed) Administered Intravenously Every 3 Weeks in Advanced Cancer Patients with Varying Degrees of Renal Function	1	1806 Results	1806 Citations
Malignant Pleural Mesothelioma	2234	A Phase 1 Clinical Trial of LY231514 in Combination with Carboplatin in Patients with Malignant Pleural Mesothelioma	1	2234 Results	2234 Citations
Malignant Pleural Mesothelioma	5249	Phase I/II Study of LY231514 and Cisplatin Combination Therapy in Patients with Malignant Pleural Mesothelioma	1 / 2	5249 Results	
Malignant Pleural Mesothelioma	3653	A Phase 2 Trial of LY231514 Administered Intravenously Every 21 Days in Patients with Malignant Pleural Mesothelioma	2	3653 Results	3653 Citations
Malignant Pleural Mesothelioma	7214	Pemetrexed Plus Gemcitabine as Front-line Chemotherapy for Patients with Malignant Pleural or Peritoneal Mesothelioma: A Phase II Clinical Trial	2	7214 Results	
Malignant Pleural Mesothelioma	2258	A Single-blind Randomized Phase 3 Trial of MTA plus Cisplatin versus Cisplatin in Patients with Malignant Pleural Mesothelioma	3	2258 Results	2258 Citations
Non-Small Cell Lung Cancer (NSCLC), 1st Line	1809	A Phase 1/2 Dose-Escalating Study Of Mta And Vinorelbine Administered Every 21 Days In Patients With Locally Advanced Or Metastatic Cancer	1 / 2	1809 Results	1809 Citations
Non-Small Cell Lung Cancer (NSCLC), 1st Line	7221	Phase 1/2 Dose-Escalating Study of Biweekly Pemetrexed and Gemcitabine in Patients with Advanced Cancer	1 / 2	7221 Results	
Non-Small Cell Lung Cancer (NSCLC), 1st Line	5114	Alimta Plus Carboplatin Or Alimta Plus Oxaliplatin As Front-Line Chemotherapy For Patients With Locally Advanced Or Metastatic Non-Small Cell Lung Cancer: A Randomized Phase 2 Clinical Trial	2	5114 Results	5114 Citations
Non-Small Cell Lung Cancer (NSCLC), 1st Line	5115	A Phase II Clinical Trial Evaluating Three Schedules of ALIMTA® Plus Gemcitabine as Front-Line Chemotherapy for Patients With Locally Advanced or Metastatic Non-Small Cell Lung Cancer	2	5115 Results	5115 Citations
Non-Small Cell Lung Cancer (NSCLC), 1st Line	5119	A Multicenter Phase 2 Randomized Trial of Single-Agent ALIMTA® or ALIMTA with Sequentially Administered GEMZAR® as First-Line Chemotherapy in Elderly Patients or Patients who are not Eligible for Platinum-Based Chemotherapy with Advanced NSCLC.	2	5119 Results	5119 Citations
Non-Small Cell Lung Cancer (NSCLC), 1st Line	6142	Alimta Plus Carboplatin as Front-Line Chemotherapy for Patients With Locally Advanced or Metastatic Non-Small Cell Lung Cancer: A Phase II Clinical Trial	2	6142 Results	6142 Citations
Non-Small Cell Lung Cancer (NSCLC), 1st Line	7211	Alimta Plus Gemcitabine as Front-Line Chemotherapy for Patients with Locally Advanced or Metastatic Non-Small Cell Lung Cancer: A Phase II Clinical Trial	2	7211 Results	7211 Citations
Non-Small Cell Lung Cancer (NSCLC), 2nd Line	1292	A Phase 1 Study of Alimta® (pemetrexed) using a daily x 5 q 21 schedule	1	1292 Results	1292 Citations
Non-Small Cell Lung Cancer (NSCLC), 2nd Line	1293	A Phase 1 Study of LY231514 Administered as a Bolus Infusion Every 21 Days	1	1293 Results	1293 Citations
Non-Small Cell Lung Cancer (NSCLC), 2nd Line	1294	A Phase 1 Study of LY231514 Administered as a Bolus Given Intravenously Every 7 Days	1	1294 Results	1294 Citations
Non-Small Cell Lung Cancer (NSCLC), 2nd Line	1790	A Phase 2 Trial of LY231514 Administered Intravenously Every 21 Days in Patients with Non-Small Cell Lung Cancer Who Have Failed Previous Chemotherapy	2	1790 Results	1790 Citations
Non-Small Cell Lung Cancer (NSCLC), 2nd Line	6685	Open-Label Single-Arm Phase 2 Study of ALIMTA in Patients with Advanced Non-Small Cell Lung Cancer Who Have Had Prior Chemotherapy	2	6685 Results	
Non-Small Cell Lung Cancer (NSCLC), 2nd Line	4881	A Phase 3 Trial of ALIMTA vs Docetaxel in Patients with Locally Advanced or Metastatic Non-Small Cell Lung Cancer (NSCLC) Who Were Previously Treated with Chemotherapy	3	4881 Results	4881 Citations
Pancreatic Cancer	8621	A Phase 2 Trial of Pemetrexed (ALIMTA®) in Pretreated Patients with Unresectable or Metastatic Cancer of the Pancreas	2	8621 Results	8621 Citations
Pancreatic Cancer	5148	A Phase 3 Trial of ALIMTA Plus GEMZAR Versus GEMZAR in Patients With Unresectable or Metastatic Cancer of the Pancreas	3	5148 Results	5148 Citations
Prostate Cancer	9772	Phase 1/2 Trial of ALIMTA in Androgen-Independent Metastatic and/or Unresectable Prostate Cancer	1 / 2	9772 Results	

Small Cell Lung Cancer	7209	Phase II Trial of ALIMTA in Relapsed Small Cell Lung Cancer	2	7209 Results	
Small Cell Lung Cancer	7210	A Randomized Phase II Trial of ALIMTA/Cisplatin and ALIMTA/Carboplatin in Extensive Stage Small Cell Lung Cancer	2	7210 Results	7210 Citations

Summary ID# 2258

Clinical Study Summary: Study H3E-MC-JMCH

Title of Study: A Single-blind Randomized Phase 3 Trial of ALIMTA (pemetrexed) plus Cisplatin versus Cisplatin Alone in Patients with Malignant Pleural Mesothelioma	
Investigator(s): This multicenter study included 88 investigators who entered patients.	
Study Center(s): A total of 88 study centers were located in 20 countries.	
Length of Study: April 1999 through February 2002	**Phase of Development:** 3

Objectives:

Primary: To compare survival in chemonaive patients with malignant pleural mesothelioma (MPM) when treated with pemetrexed plus cisplatin combination therapy with survival in the same patient population when treated with cisplatin alone.

Secondary: To compare between the two treatment arms: (1) time-to-event efficacy measures, including: a) duration of response for responding patients, b) time to progressive disease (TTPD), c) time to treatment failure (TTTF); (2) tumor response rate; (3) clinical benefit (CB) response rate; (4) Lung Cancer Symptom Scale (LCSS) patient and observer scores; (5) pulmonary function tests (PFTs); (6) lung density; (7) relative toxicities; (8) to assess the impact of folic acid and vitamin B_{12} supplementation on toxicity; (9) pharmacokinetics (PK); (10) information regarding vitamin metabolite status in this patient population.

Study Design: This study was an international, single-blind, multicenter, randomized, parallel-arm study.

Number of Patients:

A total of 574 patients were entered into the study; 456 of these patients were randomized to a treatment arm; 448 of these patients were treated and constitute the randomized and treated (RT) population.

Pemetrexed plus cisplatin: Total: 226, Male: 184, Female: 42

Fully Supplemented (FS): 168, Partially Supplemented (PS) or Never Supplemented (NS): 58

Cisplatin alone: Total: 222, Male: 181, Female: 41

Fully Supplemented (FS): 163; Partially Supplemented (PS) or Never Supplemented (NS): 59

Diagnosis and Main Criteria for Inclusion:

- MPM confirmed by histologic evaluation
- patients who were not candidates for curative surgery
- patients who had unidimensionally or bidimensionally measurable disease or both
- patients could have undergone pleurodesis if there was a 2-week delay before the administration of study drug
- performance status of ≥70 on the Karnofsky performance status (KPS) scale, after palliative measures that included pleural drainage had taken place
- estimated life expectancy of at least 12 weeks
- patient compliance and geographic proximity that allowed adequate follow-up
- adequate organ function that included the following:
  - adequate bone marrow reserve: absolute neutrophil count (ANC) $\geq 1.5 \times 10^9$/L, platelet count $\geq 100 \times 10^9$/L, and hemoglobin ≥9 g/dL
  - hepatic: bilirubin ≤1.5 times the upper limit of normal, alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) ≤3.0 times the upper limit of normal (ALP, AST, ALT ≤5 times the upper limit of normal was acceptable if there was tumor involvement in the liver). Albumin ≥2.5 g/dL
  - renal: calculated creatinine clearance (CrCl) ≥45 mL/min by using the lean body mass formula only
- signed informed consent from the patient
- male and female patients at least 18 years of age
- male and female patients with reproductive potential were required to use an approved contraceptive method during the time of and for 3 months after their participation in the study ended.

Test Product, Dose, and Mode of Administration:

Pemetrexed plus cisplatin treatment arm: pemetrexed was administered at the dose of 500 mg/m^2 as a 10-minute intravenous infusion, diluted in approximately 100 mL normal saline. Approximately 30 minutes after the administration of pemetrexed, cisplatin was administered at the dose of 75 mg/m^2 over 2 hours. Both drugs were administered on Day 1 of a 21-day period. This 21-day period defined one cycle of therapy.

Single-agent cisplatin treatment arm: approximately 100 mL normal saline was given as an intravenous infusion over approximately 10 minutes. Approximately 30 minutes after the administration of normal saline, cisplatin was administered at 75 mg/m^2 over 2 hours on Day 1 of a 21-day period. This 21-day period defined one cycle of therapy.

Both treatment arms:

- Dexamethasone, 4 mg (or an equivalent corticosteroid), was to be taken by all enrolled patients orally twice a day (BID) 1 day before, on the day of, and 1 day after each dose of pemetrexed, for primary prophylaxis against rash.
- Folic acid and vitamin B_{12} for supplementation were a standard component of therapy for all patients participating in the study from 02 December 1999 onward. Folic acid, 350 µg to 1000 µg, was to be taken orally daily, beginning approximately 1 to 3 weeks before the first dose of therapy and continued daily for 1 to 3 weeks after the patient discontinued treatment. A vitamin B_{12} injection, 1000 µg, was to be administered intramuscularly approximately 1 to 3 weeks before the first dose of therapy and should have been repeated approximately every 9 weeks until the patient discontinued study therapy.
- Pre- and posthydration for cisplatin was administered according to institutional guidelines.

Duration of Treatment: For the purposes of treating this patient population, a regimen of pemetrexed plus cisplatin or single-agent cisplatin was defined as six cycles of therapy.

Variables:

Efficacy – Survival and Time-to-Events:

All patients in the RT population were included in the analyses of survival and other time-to-event measures.

Efficacy – Tumor Response:

Enrolled patients who met the following criteria were included in the analyses of tumor response rate:

- histologic diagnosis of MPM
- no prior systemic chemotherapy
- no concurrent systemic chemotherapy or radiotherapy
- presence of unidimensionally or bidimensionally measurable disease or both
- treatment with at least one dose of pemetrexed and cisplatin (Arm A) or one dose of cisplatin (Arm B).

Efficacy – Clinical Benefit Response

Patients in the RT population who met at least one of the following criteria, and who had at least one baseline and postbaseline measurement were included in the CB response analysis:

- presence of MPM-related pain intensity at baseline as reflected by a score of ≥10 mm on a 100-mm visual analog scale (VAS)
- presence of MPM-related dyspnea at baseline as reflected by a score of ≥10 mm on a 100-mm VAS
- analgesic consumption at baseline of ≥10 mg morphine equivalents per day for MPM-related pain, and daily consumption within 50% of average baseline consumption.

Efficacy – LCSS, PFTs, Lung Density:

Enrolled patients who had at least one baseline and postbaseline measurement were included in the following analyses:

- LCSS
- PFTs
- lung density measurements.

Safety: All patients who received at least one dose of pemetrexed or cisplatin (Arm A) or one dose of cisplatin (Arm B) were evaluated for safety by assessments of exposure to study drug, treatment-emergent adverse events, serious adverse events, CTC (Version 2) toxicities for both laboratory and nonlaboratory values, and blood transfusions.

Pharmacokinetics: Pharmacokinetic parameters determined from plasma concentration versus time data included: total systemic clearance (CL), central volume of distribution (V_1), intercompartmental clearance (Q), and peripheral volume of distribution (V_2).

Evaluation Methods:

Efficacy: The primary analysis was comparison of survival time between the study arms in the RT population. Differences were assessed using a two-sided log rank test. Because an interim analysis was conducted (resulting in a decision to continue the trial to planned completion), the comparison of survival was tested at the α=0.0476 level. Comparison of survival was also tested using the Wilcoxon test.

Key secondary analyses were conducted to assess the impact of supplementation on survival in the pemetrexed/cisplatin (pem/cis) arm. The Kaplan-Meier (K-M) subgroup analyses of survival were conducted on FS and on PS+NS patients. Also, survival time was analyzed with a Cox proportional hazards model including treatment arm, supplementation group, and the treatment-by-supplementation interaction. The interaction term was evaluated to assess the impact of supplementation on the survival benefit associated with pem/cis.

Other time-to-event measures were analyzed by using the same method as described for survival time. Comparisons of the tumor response rates between the two treatment arms (in the RT, FS, and PS+NS populations) were made by using the Fishers exact (FE) test with 95% CI calculated using the method of Leemis and Trivedi. Tumor response was also analyzed with a logistic regression model including treatment arm, supplementation group, and the treatment-by-supplementation interaction. The interaction term was evaluated to assess the impact of supplementation on the survival benefit associated with pem/cis.

Time-to-event and tumor response measures were also analyzed to assess the effect of potential prognostic factors. Subgroup analyses were conducted on statistically significant factors ($p<0.05$).

Repeated measures analyses were conducted on LCSS patient scale and PFT parameters by using linear mixed models. Clinical benefit response was analyzed by using the FE test.

Safety: Adverse events and CTC toxicities were analyzed using the FE test. Between-treatment arm comparisons were made in the RT, FS, PS+NS, and NS populations. Within the pem/cis arm, comparisons were made between the FS and PS+NS subpopulation and between the FS and NS subpopulations. All parameters were analyzed as percent of patients. Selected CTC toxicities were also analyzed as a percent of cycles.

Pharmacokinetics: Blood samples were collected for pharmacokinetic (PK) analysis from the first and third cycles of therapy. The concentrations of pemetrexed in plasma were measured using a validated LC/ESI/MS/MS method. Plasma samples were analyzed for total platinum administered as cisplatin (II) using a validated atomic absorption with a tube atomizer method. Pemetrexed plasma concentration-time data from the current study were combined with a large reference dataset containing data from 8 Phase 2 single-agent pemetrexed studies. The PK analysis of plasma pemetrexed and cisplatin concentration-time data and PK comparisons were performed using population pharmacokinetic methods by means of the nonlinear mixed-effects modeling program, NONMEM. A reduction in the minimum objective function of >3.841 (with 1 df, $p<0.05$) was considered statistically significant.

Summary:

Of 574 patients who signed informed consent, 456 were considered to have fulfilled eligibility criteria for study enrollment. Of these, 448 patients received at least one course of study drug; 226 patients were allocated to receive pem/cis, and 222 were randomized to receive cisplatin alone. Reasons why 8 randomized patients did not receive study drug included patient decision (4), inclusion criteria not met (2), uncontrolled hypertension (1), and death from mesothelioma (1).

The treatment groups were well balanced in basic characteristics including gender, age, and ethnic origin (Table 1). Factors considered of potential prognostic significance were also well balanced as most were incorporated into the a priori stratification scheme.

Table 1. Patient Characteristics and Key Prognostic Factors RT Population H3E-MC-JMCH

Characteristic/Prognostic Factor	Randomized and Treated (N=448)	
	Pemetrexed/Cisplatin (N=226)	Cisplatin (N=222)
Gender		
Male	81.4%	81.5%
Female	18.6%	18.5
Age (years)		
Median	61	60
Minimum	29	19
Maximum	85	84
Ethnic origin		
Caucasian	90.3%	92.8%
Hispanic	4.9%	5.4%
Asian[1]	4.4%	1.9%
African	0.4%	0
Performance Status (KPS ≤80)	48.2%	43.7%
Stage III/IV	77.3%	78.6%
Histologic Subtype: Epithelial	68.1%	68.5%

Abbreviations: KPS = Karnofsky performance status; RT = randomized and treated.

[1] Western and East/Southeast Asian have been combined.

Prior therapy included prior radiation therapy (pem/cis 9.7% versus cisplatin alone 14.0%) and prior chemotherapy as intrapleural therapy for pleurodesis (pem/cis 7.5% versus cisplatin alone 5.0%).

Completion of six cycles of treatment was achieved in 53.1% of pem/cis treated patients compared with 40.1% of those treated with cisplatin alone. The most common reasons

for not completing six cycles included unsatisfactory response to treatment (pem/cis 27.0% versus cisplatin alone 45.5%), one or more adverse events (pem/cis 11.9% versus cisplatin alone 8.1%), patient decision or personal conflict (pem/cis 4.9% versus cisplatin alone 5.0%), and satisfactory response as perceived by patient and/or physician (pem/cis 5.3% versus cisplatin alone 1.9%).

Results:

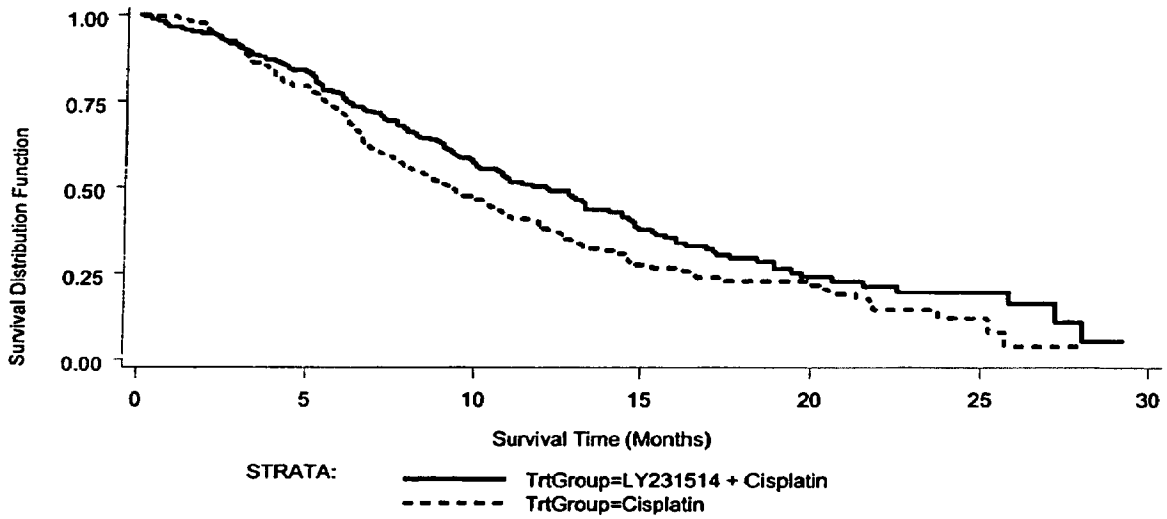
The median number of cycles given in the pem/cis arm was 6.0 cycles (range 1 to 12) versus 4.0 cycles (range 1 to 9) in the cisplatin alone arm. Within treatment arms, the median cycles delivered were higher for fully supplemented versus never supplemented patients for both arms (pem/cis: FS 6.0 versus NS 2.0; cisplatin alone: FS 4.0 versus NS 2.0). The dose intensity delivered as a percent of planned dose intensity was 92.4% for the pem/cis arm (pem 92.0% and cis 92.8%) versus 96.4% for the cisplatin alone arm; this is an indication of the very few doses reduced or omitted in either arm during the study.

The combination of pemetrexed and cisplatin was associated with a statistically significant improvement in survival (see Figure 1). Refer to Table 2 for additional findings.

Table 2. Summary of Results from Efficacy Analyses
RT Population
H3E-MC-JMCH

	Randomized and Treated (N=448)		
	Pemetrexed/Cisplatin (N=226)	Cisplatin (N=222)	Statistics
Survival	12.1 months	9.3 months	HR 0.77, p=0.020
TTPD	5.7 months	3.9 months	HR 0.68, p=0.001
TTTF	4.5 months	2.7 months	HR 0.61, p=0.001
Tumor Response Rate	41.3%	16.7%	p<0.001
Duration of Response	5.8 months	4.7 months	HR 0.82, p=0.589
PFT Improvement (as % predicted average change from baseline LS Mean over six cycles):			
• slow vital capacity	4.80	0.15	p=0.001
• forced vital capacity	4.03	-0.21	p=0.002
• forced expiratory volume in one second	3.77	-1.22	p<0.001
Patient LCSS Scores (as change from baseline LS Mean for Cycle 6) (positive change indicates worsening)			
• Fatigue	7.05	12.74	p=0.039
• Dyspnea	0.17	6.91	p=0.009
• Pain	-1.23	5.80	p=0.009
CB response rate (hybrid)	21.2%	13.6%	p=0.073

Abbreviations: CB = clinical benefit; HR = hazard ratio; LCSS = Lung Cancer Symptom Scale; LS = least squares; PFT = pulmonary function test; RT = randomized and treated; TTPD = time to progressive disease; TTTF = time to treatment failure.



Program name: ttevent4.SAS. Variable name: survtime. Population: All.

Figure 1. **Kaplan-Meier estimates of survival for RT population.**

LY231514 = pemetrexed.

Very similar results favoring the pem/cis arm were obtained when comparing the FS subgroups between treatment arms. For survival, the median survival in the pem/cis arm was 13.3 versus 10.0 months in the control arm, with an HR of 0.75, p=0.051. The median TTPD was 6.1 versus 3.9 months, HR of 0.64, with p= 0.008. Results from the other secondary outcomes support these data. Survival, TTPD, TTTF, and tumor response rate were all numerically superior in the FS subgroup compared with the PS+NS subgroup

A Cox regression analysis of factors that could affect survival and other outcomes and thus confound any treatment effect was performed. The best-fit model showed clearly that the treatment effect was independent of the presence of these factors; similar results were obtained when this model was applied to secondary efficacy outcomes such as TTPD, TTTF, and response rate. This analysis also showed that partially supplemented patients had survival characteristics that were more similar to fully supplemented patients than to never supplemented patients. In the subset of the FS+PS patient population, the median survival in the pem/cis arm was 13.2 months versus 9.4 months in the cisplatin control arm (log-rank p=0.022).

Death rates of all causes between treatment arms were similar and were further reduced with the implementation of supplementation. Only one death in the pem/cis arm was reported by investigators to be possibly study drug-related (febrile neutropenia); this patient was nonsupplemented. Two additional deaths in the pem/cis arm, before the addition of supplementation, were thought to be study drug-related in the opinion of the Lilly physician. No study drug-related deaths were reported among the pem/cis patients in the fully supplemented subgroup. By contrast, there were no study-related deaths in the control arm. The frequencies of discontinuations because of adverse events were low in both arms. More than half of the discontinuations in both arms were because of reduced creatinine clearance, suggesting a common cause as cisplatin. The remaining discontinuations thought due to study drugs were distributed over both arms, and each had a different cause.

The frequency of Grade 3 and 4 laboratory toxicity was higher in the pem/cis arm when compared with the control arm; however, clinical sequalae such as serious infection or dehydration were relatively infrequent (see Table 3). Transfusions were infrequent, and colony-stimulating factors and leucovorin were used to reverse established severe toxicity. Among the RT population, more patients on the pem/cis arm received transfusions compared with those randomized to the cis arm (18.1% versus 7.7%).

Dose reductions and dose delays were seldom necessary as reflected in a high proportion of planned doses delivered for all study drugs (>95% of planned doses were delivered). Other laboratory Grade 3 or 4 toxicities were rare, with only three episodes of Grade 4 toxicity (2 occurrences of decreased creatinine clearance and 1 occurrence of elevated glutamyl transpeptidase [GGT]).

Grade 3/4 nonlaboratory toxicity was relatively uncommon in both treatment arms (see Table 3). Nausea, vomiting, and fatigue were the most commonly reported Grade 3/4 nonlaboratory toxicities in both treatment arms. Nausea and vomiting were more frequent in the pem/cis arm despite the similar frequency of therapy with 5-HT_3 antagonists in the two arms.

Table 3. Summary of Maximum CTC Grade 3/4 Toxicity Grades RT Population H3E-MC-JMCH

Toxicity	Randomized & Treated (N=448)		
	Pemetrexed/Cisplatin (N=226)	Cisplatin (N=222)	Statistics
Selected Laboratory			
Anemia	11 (4.9%)	0	0.001
Leukopenia	40 (17.7)	2 (0.9%)	<0.001
Lymphopenia	1 (0.4)	1 (0.5)	N/A
Neutropenia	63 (27.9)	5 (2.3)	<0.001
Thrombocytopenia	13 (5.8)	0	<0.001
Selected Nonlaboratory			
Nausea	33 (14.6%)	14 (6.3%)	0.005
Vomiting	30 (13.3)	8 (3.6)	<0.001
Fatigue	23 (10.2)	19 (8.6)	0.628
Diarrhea	10 (4.4)	0	0.002
Dehydration	9 (4.0)	1 (0.5)	0.020
Stomatitis	9 (4.0)	0	0.004
Anorexia	5 (2.2)	1 (0.5)	0.216
Constitutional Symptoms – Other[a]	5 (2.2)	0	0.061
Febrile Neutropenia	4 (1.8)	1 (0.5)	0.372
Infection with G3 or G4 Neutropenia	3 (1.3)	1 (0.5)	0.623
Rash/Desquamation	3 (1.3)	0	--
Constipation	2 (0.9)	2 (0.9)	>0.999
Infection without Neutropenia	1 (0.4)	0	--

Abbreviations: CTC = Common Toxicity Criteria; G = grade; N/A = not applicable; RT = randomized and treated.

[a] Other includes dehydration not coded to GI05, hypovolemia, abdominal fullness, and worsening of general condition.

Within the pem/cis arm, supplementation resulted overall in less toxicity, including less Grade 3/4 toxicity (See Table 4); this was associated with a statistically significant increase in the median number of cycles administered in the fully supplemented subgroup. The frequencies of treatment-emergent adverse events were uniformly lower in the fully supplemented subgroup when compared to the nonsupplemented subgroup; some were significantly affected, including neutropenia, anorexia, stomatitis, and skin rash. The frequencies of all types of adverse events were also reduced in the fully supplemented subgroup. Although Grade 3/4 hematologic toxicities all decreased in the fully supplemented subgroup, this decrease was particularly striking for neutropenia and leukopenia, with the severe neutropenia rate falling nearly in half from 41% to 23%. The incidence of other nonlaboratory toxicities also decreased in the fully supplemented

subgroup, including diarrhea, stomatitis, nausea, and vomiting, with significant decrease in febrile neutropenia and infection with Grade 3 or 4 neutropenia. Exploratory analyses comparing fully supplemented with never supplemented patients showed similar results, with those never supplemented patients having the most severe toxicity in all parameters.

Supplementation was also given in the cisplatin alone arm, allowing similar comparisons as in the pem/cis arm. There was a general trend toward fewer treatment-emergent adverse events in the fully supplemented subgroup, though the differences were generally less than those seen in the pem/cis arm. For serious adverse events, there was no discernable trend toward fewer events in the fully supplemented subgroup. The numbers of Grade 3 or 4 laboratory or nonlaboratory toxicities were small in this arm, making any assessment of trends difficult and unreliable; however, in categories where the event rate was higher, there was a suggestion of fewer patients with severe nausea but more patients with severe fatigue and vomiting in the fully supplemented subgroup. Given the high rate and similar distribution of 5-HT_3 antagonist use among the subgroups in this arm, it is unclear why this discrepancy in trends should have occurred except for the fact that the event rate was small for all of these categories.

Table 4. Summary of Selected CTC Grade 3/4 Toxicities RT Population by Supplementation Status H3E-MC-JMCH

	Pemetrexed/ Cisplatin		Cisplatin	
Event Classification	FS (N=168)	PS + NS (N=58)	FS (N=163)	PS + NS (N=59)
G4 Neutrophils	9 (5.4%)	9 (15.5)	1 (0.6)	0
G3/4 Diarrhea	6 (3.6)	4 (6.9)	0	0
G3/4 Stomatitis	5 (3.0)	4 (6.9)	0	0
G4 Neutrophils + G3/4 Diarrhea	2 (1.2)	3 (5.2)	0	0
G4 Hematologic + G3/4 Non-labs	2 (1.2)	3 (5.2)	0	0
G3/4 Infection	0	3 (5.2)	0	1 (1.7)
G4 Platelet	2 (1.2)	1 (1.7)	0	0
G4 Neutrophils + G3/4 Infection	0	1 (1.7)	0	0
G4 Neutrophils + G3/4 Stomatitis	0	1 (1.7)	0	0

Abbreviations: CTC = Common Toxicity Criteria; FS = fully supplemented; G = grade; NS = never supplemented; PS = partially supplemented; RT = randomized and treated.

Among supplemented patients, homocysteine levels markedly decreased after the implementation of supplementation. Methylmalonic acid and cystathionine levels resulted in no change due to supplementation.

Concomitant cisplatin administration did not alter pemetrexed CL (change in the minimum objective function [ΔMOF] = -0.523; $p>0.05$) but was associated with a 30% reduction in V_1 (ΔMOF = -57.247; $p<001$). Oral folic acid or intramuscular vitamin B_{12} administration did not alter pemetrexed CL (ΔMOF = -0.958; $p>0.05$). Concomitant

pemetrexed administration did not significantly alter total platinum CL (ΔMOF = 0.506; $p>0.05$).

Summary of Results:

This study is the largest Phase 3 randomized trial ever conducted in MPM, and the clinical characteristics of the patients represent the typical patient population of MPM. Stage, histological type, and other prognostic factors are similar to those reported in the literature, and they were very well balanced between the two arms. This well-powered, randomized trial has demonstrated the following:

- Treatment with pemetrexed/cisplatin was superior to cisplatin monotherapy in the randomized and treated population in terms of the following endpoints:
  - longer survival
  - longer time to disease progression
  - higher tumor response rates
  - improvement in pulmonary function
  - improvement in clinically relevant symptoms commonly associated with malignant pleural mesothelioma.
- The superiority of pemetrexed/cisplatin over cisplatin monotherapy was maintained when clinically relevant prognostic factors were taken into account.
- The superiority of pemetrexed/cisplatin over cisplatin monotherapy was maintained in the fully supplemented subgroup.
- Folic acid and vitamin B_{12} supplementation improved the clinical outcome regardless of the treatment arm. The advantage was associated with more cycles delivered in the fully supplemented subgroups.
- Pemetrexed combined with cisplatin as specified in this study is associated with more toxicity compared to cisplatin alone. This toxicity was mainly hematologic with relatively few clinically significant complications such as febrile neutropenia, sepsis, or dehydration.
- Among patients receiving the pemetrexed /cisplatin combination, those who received supplementation from the start of therapy experienced less laboratory and nonlaboratory toxicity compared with patients who never received supplementation or only received it for some of their cycles of therapy.

- Patients receiving cisplatin alone showed trends toward less toxicity in those who received supplementation throughout their study treatment. But the event rate in all subgroups was low, making conclusions tenuous.
- Patients in the fully supplemented subgroups were able to receive significantly more cycles of therapy than the nonsupplemented subgroups, possibly due to the reduced toxicity associated with supplementation. This is also supported by the higher median dose intensity in the fully supplemented subgroups compared to the nonsupplemented subgroups in the pemetrexed/cisplatin arm.
- Concomitant cisplatin administration was not associated with an alteration in pemetrexed clearance but was associated with a significant reduction (30%) in central distribution volume.
- No significant influence of folic acid or vitamin B_{12} administration on pemetrexed clearance was identified.
- The population pharmacokinetics of cisplatin were assessed and found to be consistent with those previously reported in the literature.
- No significant influence of concomitant pemetrexed administration on cisplatin clearance was observed.

Alimta® (pemetrexed)

CT #2258

Title	Author	Journal	Citation
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Phase III Study of Pemetrexed in Combination With Cisplatin Versus Cisplatin Alone in Patients With Malignant Pleural Mesothelioma

By Nicholas J. Vogelzang, James J. Rusthoven, James Symanowski, Claude Denham, E. Kaukel, Pierre Ruffie, Ulrich Gatzemeier, Michael Boyer, Salih Emri, Christian Manegold, Clet Niyikiza, and Paolo Paoletti

Purpose: **Patients with malignant pleural mesothelioma, a rapidly progressing malignancy with a median survival time of 6 to 9 months, have previously responded poorly to chemotherapy. We conducted a phase III trial to determine whether treatment with pemetrexed and cisplatin results in survival time superior to that achieved with cisplatin alone.**

Patients and Methods: **Chemotherapy-naive patients who were not eligible for curative surgery were randomly assigned to receive pemetrexed 500 mg/m^2 and cisplatin 75 mg/m^2 on day 1, or cisplatin 75 mg/m^2 on day 1. Both regimens were given intravenously every 21 days.**

Results: **A total of 456 patients were assigned: 226 received pemetrexed and cisplatin, 222 received cisplatin alone, and eight never received therapy. Median survival time in the pemetrexed/cisplatin arm was 12.1 months versus 9.3 months in the control arm ($P = .020$, two-sided log-rank test). The hazard ratio for death of patients in the pemetrexed/cisplatin arm versus those in the control arm was 0.77. Median time to progression was significantly longer in the pemetrexed/cisplatin arm: 5.7 months versus 3.9 months ($P = .001$). Response rates were 41.3% in the pemetrexed/cisplatin arm versus 16.7% in the control arm ($P < .0001$). After 117 patients had enrolled, folic acid and vitamin B_{12} were added to reduce toxicity, resulting in a significant reduction in toxicities in the pemetrexed/cisplatin arm.**

Conclusion: **Treatment with pemetrexed plus cisplatin and vitamin supplementation resulted in superior survival time, time to progression, and response rates compared with treatment with cisplatin alone in patients with malignant pleural mesothelioma. Addition of folic acid and vitamin B_{12} significantly reduced toxicity without adversely affecting survival time.**

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MALIGNANT PLEURAL mesothelioma (MPM) is a locally invasive and rapidly fatal malignancy linked to asbestos exposure. Surgical resection is possible in a minority of patients, and fewer than 15% of these patients live beyond 5 years.[1-3] For those who are not treated with curative resection, the median survival duration when receiving supportive care alone has been reported as 6 months,[4,5] whereas the median survival time of 337 patients in 11 multicenter chemotherapy trials was 7 months.[6] Treatment with radiation therapy has been equally disappointing, in part because of difficulties in irradiating disease while avoiding toxicity to normal lung, cardiac, and spinal cord tissues.[7,8]

Numerous single agents, such as cisplatin, doxorubicin, and gemcitabine, and drug combinations, such as gemcitabine and cisplatin, have been studied in phase II trials.[9-14] However, the strength of this evidence has not supported the standard use of chemotherapy. The few published randomized trials in MPM have shown negative results, have often been underpowered, and have been associated with median survival times of only 6 to 8 months.[15-19]

Recently, pemetrexed, a novel multitargeted antifolate,[20] has shown modest activity as a single agent in a phase II trial of patients with MPM (response rate, 14.1%, or nine of 64 patients).[21] Pemetrexed inhibits dihydrofolate reductase, thymidylate synthase, and glycinamide ribonucleotide formyltransferase, enzymes involved in purine and pyrimidine synthesis.[22,23] Pemetrexed enters the cell primarily through the reduced folate carrier, and undergoes extensive intracellular polyglutamation by folylpoly-gamma-glutamate synthetase. The polyglutamated forms, retained for long periods within the cell,[24] have more than 100-fold greater affinity for thymidylate synthase and glycinamide ribonucleotide formyltransferase than the parent drug, pemetrexed monoglutamate.[25] In addition to single-agent activity, responses were seen in MPM patients in two phase I trials of pemetrexed combined with platinum analogs.[26,27] In the first study of 40 assessable patients, 11 patients were enrolled with a diagnosis of MPM and were given pemetrexed combined with cisplatin, at increasing doses of both drugs. Surprisingly, five (45%) of 11 patients had a partial response (PR). The maximum-tolerated dose over all cycles was established at pemetrexed 600 mg/m^2 and cisplatin 75 mg/m^2. At this dose, seven of 12 patients experience grade 3 or 4 neutropenia, whereas eight patients experienced grade 3 or 4 anemia. This was in contrast to only one of three patients with grade 3 neutropenia or grade 4 anemia treated at the recommended phase II dose of

From the University of Chicago Cancer Research Center, Chicago, IL; Eli Lilly and Company, Indianapolis, IN; US Oncology, Dallas, TX; Allgemeines Krankenhaus Harburg, Hamburg; Krankenhaus Großhansdorf, Großhansdorf; and Thoraxklinik-Rohrbach, Heidelberg, Germany; Institut Gustave Roussy, Villejuif, France; Royal Prince Alfred Hospital, Camperdown, Australia; and Hacettepe University Medical Faculty, Ankara, Turkey.

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Address reprint requests to Nicholas J. Vogelzang, MD, University of Chicago, Cancer Research Center, 5841 South Maryland Ave, Chicago, IL 60637; email: nvogelza@medicine.bsd.uchicago.edu.

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pemetrexed 500 mg/m^2 and cisplatin 75 mg/m^2. The second trial enrolled 25 chemotherapy-naive patients with MPM who received increasing doses of both pemetrexed and carboplatin; eight patients (32%) assessable for response experienced a PR.

Encouraged by these results and by early results of a phase II trial of pemetrexed 500 mg/m^2 and cisplatin 75 mg/m^2 in patients with non–small-cell lung cancer showing that the combination at this dose was well tolerated,[28] we initiated a large, phase III clinical trial to determine whether pemetrexed/cisplatin therapy was associated with superior survival duration compared with cisplatin alone in the treatment of patients with MPM.

PATIENTS AND METHODS

Patients

Patients with histologically proven pleural mesothelioma who were not candidates for curative surgery were assessed for eligibility. Eligibility requirements included uni- or bidimensionally measurable disease, age ≥18 years with life expectancy ≥12 weeks, and a Karnofsky performance status of ≥ 70. Patients were excluded if they had prior chemotherapy, a second primary malignancy, or brain metastases, or if they were unable to interrupt nonsteroidal anti-inflammatory drugs.

Study Design

This study was a multicenter, randomized, single-blind study comparing treatment with pemetrexed and cisplatin versus cisplatin alone in MPM patients. The primary outcome was survival. Secondary outcomes reported here include time to progressive disease, time to treatment failure, tumor response rate, and duration of response. Pulmonary function testing, lung density analysis, and quality-of-life outcomes will be reported in separate publications. After informed consent was obtained, eligible patients were randomly assigned to arms of pemetrexed and cisplatin or cisplatin alone. Patient randomization was balanced for the following baseline factors: treatment center, country, pain level at entry, analgesic consumption at entry, dyspnea at entry, performance status, degree of measurability of disease, histologic subtype, sex, baseline WBC count, and baseline serum homocysteine levels.

Three treatment-related deaths (7%) were reported among the first 43 patients randomly assigned to the experimental arm. Severe toxicities (eg, grade 4 neutropenia and diarrhea) in other pemetrexed studies were linked to high blood levels of homocysteine and methylmalonic acid, at study entry, in a large multivariate analysis, suggesting that such toxicity and possibly some deaths may be related to reduced folic acid and vitamin B_{12} pools.[29] Therefore, beginning December 2, 1999, folic acid and vitamin B_{12} supplementation was required for all patients receiving pemetrexed and for those subsequently enrolled in this study. This change resulted in three patient subgroups that were defined by supplementation status: (1) never supplemented patients (NS) completed treatment before the protocol change (ie, December 2, 1999); (2) partially supplemented patients (PS) began treatment before this date and completed treatment after that date; (3) fully supplemented patients (FS) began treatment after that date. To ensure adequate statistical power of the FS subgroup, the sample size was substantially increased (see statistical plan that appears later).

Treatment

Pemetrexed was administered intravenously (IV) at 500 mg/m^2 over 10 minutes, followed 30 minutes later by cisplatin 75 mg/m^2 IV over 2 hours on day 1 of a 21-day cycle. Patients assigned to the cisplatin arm were treated likewise, except normal saline was given in the place of pemetrexed at equivalent volume. Folic acid 350 to 1,000 μg was taken orally daily beginning 1 to 3 weeks before the first chemotherapy doses and was continued throughout study therapy. Vitamin B_{12} 1,000 μg was given intramuscularly 1 to 3 weeks before the first dose of study therapy and repeated every 9 weeks while a patient was receiving study therapy. In addition, dexamethasone was given the day before, day of, and day after pemetrexed dosing to reduce the risk of severe skin rash. Both vitamin supplementation and dexamethasone were given to patients in both arms to maintain patient blinding to study therapy. Other chemotherapy, immunotherapy, or hormonal therapy was not permitted. Supportive care therapies were allowed per protocol during the study.

Dose adjustments for hematologic toxicity were based on a stepwise reduction schedule. Grade 3 or 4 mucositis, diarrhea requiring hospitalization, or grade 3 or 4 nonhematologic effects also resulted in dose reduction for subsequent doses. Any patient requiring three dose reductions was discontinued from the study. Dose delays up to 42 days were permitted for recovery from study drug toxicity. Dose escalations were not allowed.

Assessments During and After Treatment

Baseline and predosing assessment included a complete history and physical examination, complete blood cell count, calculated creatinine clearance, liver enzymes, blood electrolytes, blood albumin, calcium and glucose, and vitamin metabolites. Survival was defined as the time from randomization to the time of death from any cause. Patients who were alive on the date of last follow-up were censored on that date. Time to progressive disease was defined as the time from randomization until documented progression or death from any cause. For patients without progressive disease at the time of analysis, the date of last follow-up was considered right-censored. Duration of tumor response was defined as the time from the first objective status of a response to the time of documented disease progression or death from any cause. Chest imaging was performed at least once just before every other treatment while a patient was receiving study therapy and approximately every 6 weeks after completion of study therapy. Time to treatment failure was defined as the time from randomization to the date of observed disease progression, death from any cause, or early discontinuation of treatment.

Change in disease was assessed by measuring the thickness of up to three involved areas of pleural rind at each of three separate levels at least 2 cm apart on computed tomography scan, at baseline, and every other cycle (at least one measurement was > 1.5 cm).[30] A complete response (CR) was defined as complete absence of measurable, nonmeasurable but assessable, and unassessable disease with no new lesions and no disease-related symptoms. A PR was defined as ≥ 50% reduction from baseline of the sum of the products of perpendicular diameters of bidimensionally measurable disease when only such disease was present, ≥ 30% decrease in the sum of the greatest diameters of unidimensionally measurable lesions when only such disease was present, or reduction of either type of disease as defined above and the other type at least stable when both types were present, with nonmeasurable lesions being at least stable, with no new lesions. Any CR or PR required confirmation 4 weeks later. Tumor response rate was defined as the proportion of patients who experienced either a CR or PR times 100. Tumor progression was defined as the appearance of a new or relapsed lesion/site, a 50% increase in the sum of products of all bidimensionally measurable lesions over the smallest sum observed when only such disease was present, a 25% increase in the sum of the longest dimension of unidimensionally measurable lesions over smallest sum observed when only such disease was present (in the presence of both disease types, progression of either type as defined above and at least stable disease for the other), worsening of assessable disease, or death from disease. Stable disease was disease that did not qualify for CR, PR, or progression.

Statistical Analyses

The primary statistical analysis compared survival times between the two study arms. This primary analysis was conducted on an intent-to-treat (ITT) basis. Secondary analyses were conducted comparing subgroups defined by supplementation status within or across treatment arms to assess the effect of supplementation on safety and efficacy. Unless otherwise noted, all tests of hypotheses were conducted at the alpha = 0.050 level, with a 95% confidence interval.

Kaplan-Meier nonparametric techniques[31] were used for the comparison of survival times between the two treatment arms in the ITT population. Differences were assessed using a two-sided log-rank test. Because an interim analysis was conducted (resulting in a decision to continue the trial

Table 1. Patient Characteristics

	Pemetrexed/Cisplatin				Cisplatin			
	Intent to Treat (n = 226)	Full Supplementation (n = 168)	Partial Supplementation (n = 26)	Never Supplemented (n = 32)	Intent to Treat (n = 222)	Full Supplementation (n = 163)	Partial Supplementation (n = 21)	Never Supplemented (n = 38)
Age, years								
Median	61	60	62.5	61	60	60	62	59.5
Range	29-85	29-85	38-75	32-77	19-84	19-82	36-81	35-84
Sex								
Male								
No. of patients	184	136	22	26	181	134	18	29
%	81.4	81.0	84.6	81.3	81.5	82.2	85.7	76.3
Female								
No. of patients	42	32	4	6	41	29	3	9
%	18.6	19.0	15.4	18.8	18.5	17.8	14.3	23.7
Race								
White								
No. of patients	204	150	23	31	206	153	19	34
%	90.3	89.3	88.5	96.9	92.8	93.9	90.5	89.5
Other*								
No. of patients	22	18	3	1	16	10	2	4
%	9.7	10.7	11.5	3.1	7.2	6.1	9.5	10.5
Performance status								
70								
No. of patients	37	25	3	9	31	22	3	6
%	16.4	14.9	11.5	28.1	14.0	13.5	14.3	15.8
80								
No. of patients	72	58	7	7	66	47	7	12
%	31.9	34.5	26.9	21.9	29.7	28.8	33.3	31.6
90/100								
No. of patients	117	85	16	16	125	94	11	20
%	51.8	50.6	61.5	50.0	56.3	57.7	52.4	52.6
Histology								
Epithelial								
No. of patients	154	117	18	19	152	113	14	25
%	68.1	69.6	69.2	59.4	68.5	69.3	66.7	65.8
Sarcomatoid								
No. of patients	18	14	2	2	25	17	3	5
%	8.0	8.3	7.7	6.3	11.3	10.4	14.3	13.2
Mixed cell								
No. of patients	37	25	4	8	36	25	4	7
%	16.4	14.9	15.4	25.0	16.2	15.3	19.0	18.4
Unspecified								
No. of patients	17	12	2	3	9	8	0	1
%	7.5	7.1	7.7	9.4	4.1	4.9	0.0	2.6
Stage								
I								
No. of patients	16	15	1	0	14	12	0	2
%	7.1	8.9	3.8	0.0	6.3	7.4	0.0	5.3
II								
No. of patients	35	27	5	3	33	27	2	4
%	15.6	16.2	19.2	9.4	15.0	16.8	9.5	10.5
III								
No. of patients	73	51	12	10	68	49	9	10
%	32.4	30.5	46.2	31.3	30.9	30.4	42.9	26.3
IV								
No. of patients	102†	75†	8	19	107†	75†	10	22
%	45.1	44.6	30.8	59.4	48.2	46.0	47.6	57.9

*Includes Hispanics, Asians, and patients of African descent.

†Includes patients with unspecified stage (one patient in pemetrexed/cisplatin arm and two patients in cisplatin arm).

to planned completion), the comparison of survival times was tested at the $\alpha = .0476$ level. To assess the impact of supplementation on survival times in the pemetrexed/cisplatin arm, the Kaplan-Meier analysis of survival time was conducted on FS and on FS + PS patients. Statistical analyses of time-to-event secondary efficacy variables were comparable to those of the primary efficacy variable. Comparisons of the tumor response rates between the two treatment arms was made using the Fisher's exact test with 95% CIs calculated using the method of Leemis and Trivedi.[32] Dose-intensity (DI) was calculated as mean dose in milligrams per square meter per week. The percentage of planned DI delivered was calculated as the mean DI delivered in milligrams per square meter per week divided by the planned DI in milligrams per square meter per week times 100. The incidence of common toxicity criteria toxicities was analyzed using Fisher's exact test.

Table 2. Summary of Study Drug Administration

	Pemetrexed/Cisplatin				Cisplatin			
	Intent to Treat (n = 226)	Full Supplementation (n = 168)	Partial Supplementation (n = 26)	Never Supplemented (n = 32)	Intent to Treat (n = 222)	Full Supplementation (n = 163)	Partial Supplementation (n = 21)	Never Supplemented (n = 38)
Cycles given								
Median	6.0	6.0	6.0	2.0	4.0	4.0	6.0	2.0
Range	1-12	1-12	2-6	1-6	1-9	1-9	2-6	1-6
% Completing at least four cycles	71.2	73.2	96.1	40.6	55.4	55.2	85.7	39.5
% Completing at least six cycles	53.1	57.7	65.4	18.8	40.1	40.5	66.7	23.7
% Completing at least eight cycles	5.3	7.1	0	0	2.3	3.1	0	0
Dose delivered, pemetrexed								
DI, mg/m²/wk	153.4	154.6	141.3	156.6	N/A	N/A	N/A	N/A
% Planned DI	92.0	92.8	84.8	94.0	N/A	N/A	N/A	N/A
Dose delivered, cisplatin								
DI, mg/m²/wk	23.2	23.4	21.5	23.5	24.1	24.1	23.9	24.3
% Planned DI	92.8	93.6	86.0	94.0	96.4	96.4	95.6	97.2

Abbreviations: DI, dose-intensity; N/A, not applicable.

RESULTS

Patient Characteristics

From April 1999 to March 2001, 574 patients signed informed consent, and of 456 eligible patients, 226 received pemetrexed/cisplatin, and 222 received cisplatin alone. (Eight randomly assigned patients went off study before receiving any study drug; reasons were patient decision [four patients], inclusion criteria not met [two patients], hypertension [one patient],and death from study disease [one patient]). These 448 patients were assessable for efficacy and toxicity as the ITT population.

As seen in Table 1, treatment arms were well balanced with respect to baseline characteristics. Patients were predominantly male and white, with a median age of 61 years (range, 19 to 85 years). Approximately two thirds of the patients had epithelial histology, whereas 78% had stage III or stage IV disease. The most common metastatic sites included pleural rind, mediastinal lymph node, lung, and chest wall. No patient had prior systemic chemotherapy, but 12% of patients had prior radiotherapy. Pemetrexed/cisplatin patients received more treatment cycles (median, six cycles; range, one to 12 cycles) than those receiving cisplatin alone (median, four cycles; range, one to nine cycles; Table 2). Similarly, within each arm, supplemented patients received more cycles than never-supplemented patients. The delivered DI of study drugs was highly efficient, exceeding 90% in both arms. Median follow-up was 10.0 months.

Efficacy

Survival curves of the ITT population and FS subgroup for each arm are shown in Figure 1A and 1B, respectively. The median survival time for pemetrexed/cisplatin-treated patients was longer than for patients receiving cisplatin alone: 12.1 months versus 9.3 months, representing a highly statistically significant difference (Table 3). In the FS subgroup, median survival time was 13.3 months for the pemetrexed/cisplatin arm and 10.0 months in the control arm, representing a difference that approaches statistical significance ($P = .051$). Although the PS-only subgroup was a relatively small subset, comparison of this subgroup between the two arms showed a hazard ratio of 0.78, which was comparable to that of the FS subgroups. We therefore combined these subgroups to explore the effect of treatment on the subgroup of patients who received supplementation at some time during their therapy (ie, FS/PS). As can be seen in Table 3, the comparison of survival time between the two arms showed a similar treatment effect: 13.2 months for the pemetrexed/cisplatin arm versus 9.4 months for the control arm ($P = .022$). However, in the NS subgroup, there was no statistically significant difference between the two arms; this was likely due at least in part to the small numbers of patients in each subgroup (data not shown).

As with survival duration, the median time to progressive disease was significantly longer for patients who received pemetrexed and cisplatin as compared with patients who received cisplatin alone (5.7 months *v* 3.9 months; $P = .001$; Fig 2A, Table 3). This difference was similar for both the FS and FS/PS subgroups as well (Fig 2B, Table 3). The median time to treatment failure was also significantly longer in the pemetrexed/cisplatin arm than in the control arm. Again, the results were similar in the FS and FS/PS subgroups. The response rates are listed in Table 3. All responses were PRs: 41.3% of pemetrexed/cisplatin patients versus 16.7% in the control group. This magnitude of effect was similar in the vitamin-supplemented subgroups.

Toxicity

Hematologic toxicities are summarized as worst grade 3 or 4 toxicity in Tables 4 and 5. In the control arm, severe toxicity was uncommon. In the pemetrexed/cisplatin arm, grade 3/4 neutropenia (27.9%) and grade 3/4 leukopenia (17.7%) were the most common hematologic toxicities. Toxicity within this arm was analyzed comparing supplementation subgroups in two ways (ie, FS *v* combined PS/NS patients and FS/PS combined *v* NS patients; Table 5). The incidence of grade 3/4 neutropenia was significantly higher among NS/PS patients (41.4%) compared with FS patients (23.3%; $P = .011$); this difference was similar when PS/FS patients were compared with NS patients. A similar but nonsignificant trend was observed for leukopenia: 25.8% for PS/NS patients

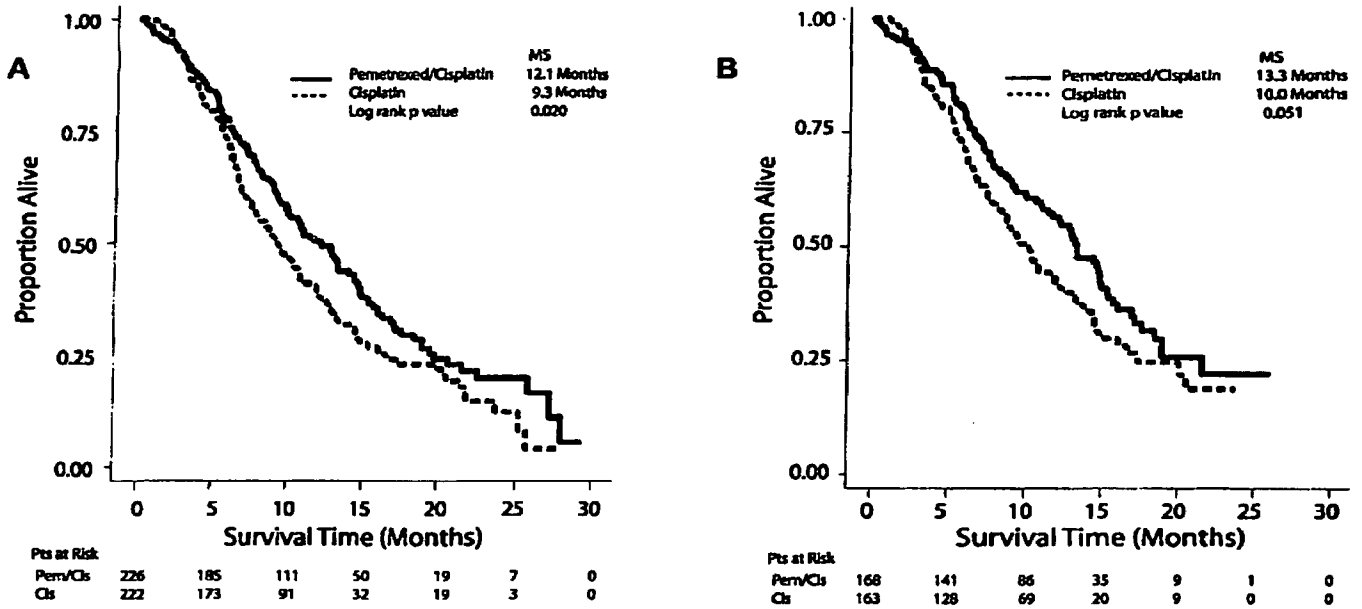

Fig 1. Kaplan-Meier estimates of overall survival time for all patients (Pts) (A) and for fully supplemented patients (B). Overall survival was significantly longer for the pemetrexed/cisplatin-treated patients (Pem/Cis) in the group of all patients (P = .020) and approached significance for the group of fully supplemented patients (P = .051). MS, median survival; Cis, cisplatin alone.

versus 14.9% for FS patients (P = .072). Nonhematologic laboratory toxicity was infrequent, with five episodes of decreased creatinine clearance and three episodes of hyponatremia, all in pemetrexed/cisplatin patients (data not shown).

Clinical toxicities are also listed in Tables 4 and 5. In both treatment groups, nausea, vomiting, and fatigue were the most commonly reported nonlaboratory toxicities, with ≥ 88% of events reported as grade 3. The incidence of nausea, vomiting,

Table 3. Results From Analysis of Efficacy Parameters

	Intent to Treat		Fully Supplemented		Fully and Partially Supplemented	
	Pemetrexed/Cisplatin (n = 226)	Cisplatin (n = 222)	Pemetrexed/Cisplatin (n = 168)	Cisplatin (n = 163)	Pemetrexed/Cisplatin (n = 194)	Cisplatin (n = 184)
Survival						
Median, months	12.1	9.3	13.3	10.0	13.2	9.4
95% CI for median	10.0 to 14.4	7.8 to 10.7	11.4 to 14.9	8.4 to 11.9	10.9 to 14.8	8.4 to 11.6
Hazard ratio	0.77		0.75		0.71	
Log-rank P	.020		.051		.022	
Wilcoxon P	.028		.039		.019	
1-year survival, %	50.3	38.0	56.5	41.9	54.1	40.9
P*	.012		.011		.014	
Percent censored	35.8	28.4	43.5	36.8	41.2	33.2
Time to PD						
Median, months	5.7	3.9	6.1	3.9	6.1	4.3
95% CI for median	4.9 to 6.5	2.8 to 4.4	5.3 to 7.0	2.8 to 4.5	5.4 to 6.7	3.0 to 4.9
Hazard ratio	0.68		0.64		0.70	
Log-rank P	.001		.008		.003	
Wilcoxon P	< .001		< .001		< .001	
Percent censored	7.5	9.0	8.9	12.3	8.8	10.9
Tumor response†						
Response rate, %	41.3	16.7	45.5	19.6	45.6	19.0
95% CI for response rate	34.8 to 48.1	12.0 to 22.2	37.8 to 53.4	13.8 to 26.6	38.4 to 52.9	13.6 to 25.4
Fisher's exact P	< .001		< .001		< .001	

Abbreviation: CI, confidence interval; PD, progressive disease.

*Two-sided P value based on standard normal distribution.

†One pemetrexed/cisplatin patient did not have measurable disease at baseline and was excluded from analysis of tumor response rate.

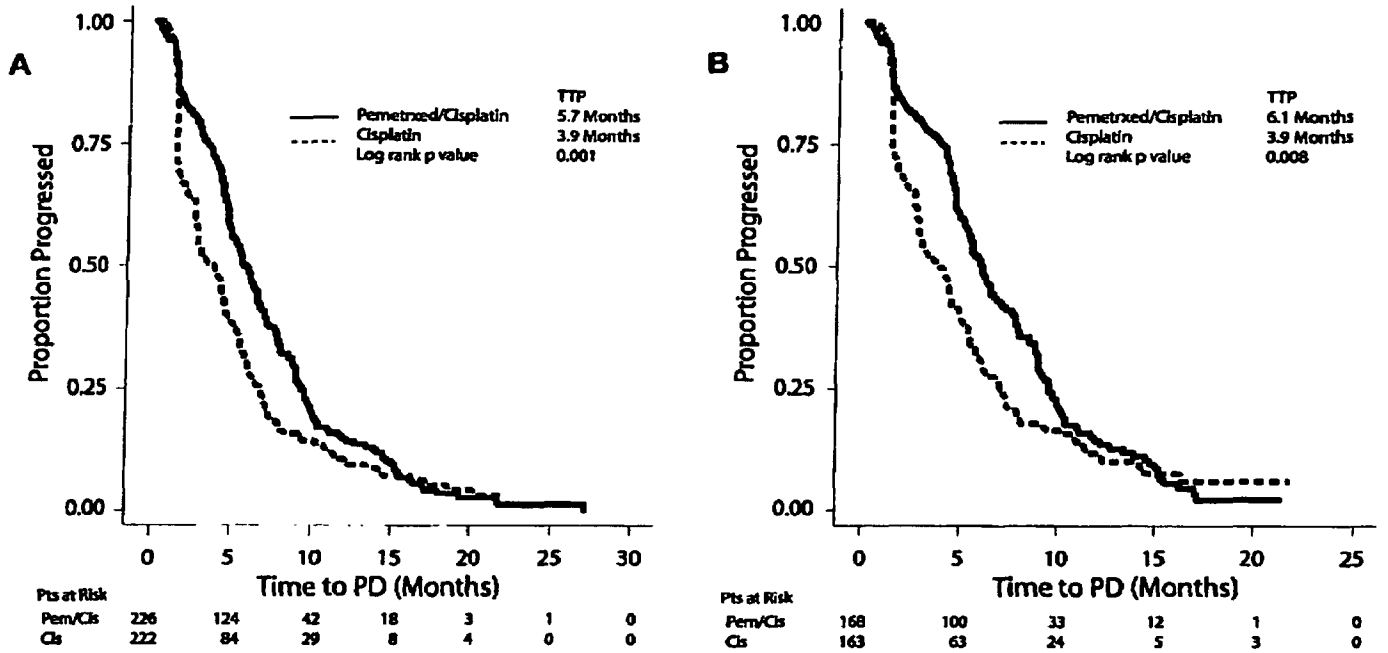

Fig 2. Kaplan-Meier estimates of time to progressive disease (PD) for all patients (Pts) (A) and for fully supplemented patients (B). Time to progressive disease was significantly longer for pemetrexed/cisplatin-treated patients (Pem/Cis) in the group of all patients ($P = .001$) and in the group of fully supplemented patients ($P = .008$). TTP, time to progression; Cis, cisplatin alone.

fatigue, diarrhea, dehydration, and stomatitis were significantly higher in the pemetrexed/cisplatin arm. In the pemetrexed/cisplatin arm, the FS subgroup experienced consistently less toxicity (except for dehydration), including less than a 1% incidence of febrile neutropenia. The FS/PS subgroup showed a similar reduction in toxicity, with differences in nausea, vomiting, and febrile neutropenia reaching statistical significance.

Fourteen patients receiving pemetrexed/cisplatin died while on study therapy or within 30 days of the last dose of study drug, compared with eight patients receiving cisplatin alone (6.2% *v* 3.6%). Three deaths thought to be at least possibly study drug-related occurred in the pemetrexed/cisplatin arm before adding vitamin supplementation; none occurred thereafter. The remaining deaths were thought to be disease-related.

DISCUSSION

This multicenter phase III study demonstrated a statistically significant improvement in survival time in MPM patients treated

Table 4. Summary of Maximum Common Toxicity Criteria Grade 3/4 Toxicities

	Pemetrexed/Cisplatin, Intent to Treat (n = 226)		Cisplatin, Intent to Treat (n = 222)		
	No. of Patients	%	No. of Patients	%	*P*
Hematologic laboratory toxicity					
Hemoglobin	11	4.8	0	0	.001
Leukocytes	40	17.7	2	0.9	< .001
Neutrophils	63	27.9	5	2.3	< .001
Platelets	13	5.8	0	0	< .001
Nonlaboratory toxicity					
Nausea	33	14.6	14	6.3	.005
Fatigue	23	10.2	19	8.6	.628
Vomiting	30	13.3	8	3.6	.000
Diarrhea	10	4.4	0	0	.002
Dehydration	9	4.0	1	0.5	.020
Stomatitis	9	4.0	0	0	.004
Anorexia	5	2.2	1	0.5	.216
Febrile neutropenia	4	1.8	0	0	.123
Infection with G3 or G4 neutropenia	3	1.3	1	0.5	.623
Rash	3	1.3	0	0	.248

*Fisher's exact *P* value for comparison of intent-to-treat pemetrexed and cisplatin group versus intent-to-treat cisplatin group.

Table 5. Summary of Maximum Common Toxicity Criteria Grade 3/4 Toxicities From Pemetrexed/Cisplatin-Treated Patients

	Full Supplementation (n = 168)		Partial Supplementation + Never Supplemented (n = 58)			Full Supplementation + Partial Supplementation (n = 194)		Never Supplemented (n = 32)		
	No. of Patients	%	No. of Patients	%	P*	No. of Patients	%	No. of Patients	%	P*
Hematologic Laboratory Toxicity										
Hemoglobin	7	4.2	4	6.9	.479	8	4.1	3	9.4	.192
Leukocytes	25	14.9	15	25.9	.072	29	14.9	11	34.4	.012
Neutrophils	39	23.2	24	41.4	.011	51	26.3	12	37.5	.205
Platelets	9	5.4	4	6.9	.744	10	5.2	3	9.4	.403
Nonlaboratory Toxicity										
Nausea	20	11.9	13	22.4	.082	23	11.9	10	31.3	.012
Fatigue	17	10.1	6	10.3	.999	18	9.3	5	15.6	.338
Vomiting	18	10.7	12	20.7	.071	20	10.3	10	31.3	.003
Diarrhea	6	3.6	4	6.9	.284	7	3.6	3	9.4	.154
Dehydration	7	4.2	2	3.4	.999	7	3.6	2	6.3	.619
Stomatitis	5	3.0	4	6.9	.240	8	4.1	1	3.1	.999
Anorexia	2	1.2	3	5.2	.108	3	1.5	2	6.3	.148
Febrile neutropenia	1	0.6	3	5.2	.053	1	0.5	3	9.4	.009
Infection with G3 or G4 neutropenia	0	0	3	5.2	.016	1	0.5	2	6.3	.053
Rash	1	0.6	2	3.4	.163	3	1.5	0	0.0	.999

*Fisher's exact P value for within-pemetrexed/cisplatin arm comparisons for the full supplementation versus partial supplementation plus never supplemented subgroups and for the full supplementation plus partial supplementation versus never supplemented subgroups.

with pemetrexed/cisplatin compared with cisplatin alone. This improvement is also clinically relevant; the additional survival time of 2.8 months in the pemetrexed/cisplatin arm is nearly twice as long as the 6-week median survival improvement found in meta-analyses and used to justify recommendations for the use of cisplatin-containing regimens in advanced non–small-cell lung cancer.[33,34] The 2.8-month survival benefit represents a hazard ratio of 0.77 or relative risk reduction for death of 23%. A risk reduction of this magnitude is usually considered a meaningful incremental survival-time improvement in oncology trials. Design features such as the large sample size and multiple strata of prognostic factors in the randomization scheme gives added confidence that this result is robust, generalized, and attributable mainly, if not solely, to the addition of pemetrexed to the treatment regimen. In addition, the presence of a high percentage of patients with advanced disease stage (III/IV) and a median survival time in the control arm that exceeded literature-based expectations,[4,5] adds to the credibility of the results. Data from two other randomized MPM trials have been reported. Samson et al reported the results of a randomized intergroup trial of cyclophosphamide, imidazole carboxamide, and doxorubicin versus cyclophosphamide and doxorubicin.[17] The sample size was underpowered (n = 76), but there was no significant difference in survival or duration of response. A second randomized trial of ranpirnase versus doxorubicin was recently reported as an abstract.[19] That trial enrolled 154 patients, and the median survival time was not significantly different in the two arms (7.7 months in the ranpirnase group and 8.2 months in the doxorubicin group).

Other antifolates (trimetrexate [response rate, 12%],[35] edatrexate [response rate, 18% and 25%],[36] and methotrexate [response rate, 37%][37]) have been tested in single-agent, phase II studies of patients with MPM. Although these studies suggest that other antifolate drugs may have some activity against pleural mesothelioma, they have not been tested in randomized trials as single agents or combinations against appropriate contemporaneous control groups. As such, the evidence supporting the use of other antifolates, in practice, remains weak. Interestingly, antitumor activity may be mediated through a newly identified class of high affinity alpha-folate receptors found on mesothelioma cells of all histologic subtypes.[38]

In addition to examining MPM treatment regimens, this study also looked at the effect of vitamin supplementation on those regimens. Patients receiving pemetrexed/cisplatin with vitamins had greater improvement in all efficacy parameters than those receiving the same regimen without vitamins. Surprisingly, patients receiving cisplatin alone also seemed to benefit from the vitamin supplementation, though to a lesser degree. Supplementation enabled patients to receive more cycles of treatment (Table 2), and this may explain these results. Most importantly, there was no adverse effect of vitamin supplementation on efficacy because the results of survival and other time-to-event outcomes consistently favored the pemetrexed/cisplatin therapy.

The overall toxicity and response profile of pemetrexed/cisplatin seemed to be similar to or better than that reported with other two-drug chemotherapeutic regimens studied in patients with MPM. However, a phase III study comparing this regimen to another widely used regimen, such as gemcitabine/cisplatin[10,11] would be necessary to clarify that hypothesis. The primary toxicity profile of pemetrexed (mucositis, neutropenia, and leukopenia) does not overlap that of cisplatin (gastrointestinal, neurological, and renal), thus supporting their use in combination. Patients who received vitamin supplementation had a notable reduction in hematologic toxicity, specifically grade 3/4 neutropenia and leukopenia, an improvement in clinical toxicity. Overall improvement in severe toxicity has been observed in other pemetrexed studies because vitamin supplementation became a standard of pemetrexed therapy.[29]

This study had some limitations. Although crossover of control patients to pemetrexed was not permitted, second-line therapy was not controlled in this trial. As a result, 37.6% of

patients on the pemetrexed/cisplatin arm and 47.3% on the control arm received second-line chemotherapy. Despite the potential risk for survival to be preferentially extended in the control arm because of its higher frequency of second-line therapy, the observed treatment effect remained statistically and clinically significant in favor of pemetrexed/cisplatin. End points, such as time to progressive disease and time to treatment failure, are unlikely to be influenced by second-line treatment, yet these outcomes were also significantly improved by pemetrexed/cisplatin. Another limitation was the lack of a double-blind design, because outcome measurements of response and time to progression could be biased by prior investigator knowledge of the treatment assignment. The response rates for both arms were as good or better than those published in most other single-agent and combination phase II studies, a result possibly influenced by such a bias or by the measurement method used in this study.

In conclusion, pemetrexed/cisplatin therapy was associated with significantly improved survival time and with overall greater antitumor activity compared with cisplatin alone. The regimen was well tolerated, particularly in patients who received low-dose folic acid and vitamin B_{12}. Vitamin supplementation reduced toxicity with no apparent adverse affect on efficacy.

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APPENDIX

The appendix is included in the full text version of this article only, available on-line at www.jco.org. It is not included in the PDF version.

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Summary ID# 3653

Clinical Study Summary: Study H3E-MC-JMDR

Title of Study: A Phase 2 Trial of LY231514 Administered Intravenously Every 21 Days in Patients with Malignant Pleural Mesothelioma	
Investigator(s): This multicenter study included 10 investigator(s).	
Study Center(s): This study was conducted at 10 study center(s) in four countries.	
Length of Study: 1 year and 10.5 months Date first patient enrolled: 01 September 1999 Date last patient completed: 14 July 2001	**Phase of Development:** 2
Objectives: The primary objective was to determine the tumor response rate of patients with malignant pleural mesothelioma who had been treated with pemetrexed. The secondary objectives were (1) to measure the time-to-event efficacy endpoints of overall survival, time to progressive disease, duration of response for responding patients, time to treatment failure; (2) to determine clinical benefit response rate after treatment with pemetrexed by using a clinical benefit response algorithm for assessments of performance status, reported pain intensity, analgesic consumption, and dyspnea; (3) to evaluate changes in Lung Cancer Symptom Scale (LCSS) scores, pulmonary function test (PFT) measures, lung density determinations; (4) to characterize the quantitative and qualitative toxicities of pemetrexed when administered once every 21 days to patients with malignant pleural mesothelioma; and (5) to evaluate the vitamin deficiency marker status of patients.	
Study Design: This was an open-label, two-stage, outpatient, Phase 2 study of pemetrexed conducted in chemonaive patients with malignant pleural mesothelioma. Folic acid and vitamin B_{12} for supplementation were a standard component of pemetrexed therapy for all patients participating in the study from 10 December 1999 onward.	
Number of Patients: Planned: 61 Enrolled: 64; Supplemented: 43; Nonsupplemented: 21.	
Diagnosis and Main Criteria for Inclusion: The main inclusion criterion was a histologic diagnosis of unidimensionally and/or bidimensionally measurable malignant pleural mesothelioma in male or female patients age 18 or older who were not candidates for curative surgery.	
Test Product, Dose, and Mode of Administration: Pemetrexed, 500 mg/m^2, was administered to all enrolled patients as a 10-minute intravenous infusion on Day 1 of a 21-day period. Dexamethasone, 4 mg (or an equivalent corticosteroid), was to be taken by all enrolled patients orally twice a day 1 day before, on the day of, and 1 day after each dose of pemetrexed, for primary prophylaxis against rash. Folic acid, 350 to 1000 µg, was to be taken orally daily, beginning approximately 1 to 2 weeks before the first dose of pemetrexed and continued daily for 1 to 2 weeks after the patient discontinued treatment. A vitamin B_{12} injection, 1000 µg, was to be administered intramuscularly approximately 1 to 2 weeks before the first dose of pemetrexed and repeated approximately every 9 weeks until the patient discontinued study therapy.	

Duration of Treatment: Multiple cycles of pemetrexed therapy could be administered. Cycles could be repeated until there was evidence of disease progression or unacceptable toxicity, or the patient requested discontinuation of therapy. Study therapy could also be discontinued if the investigator felt that it was not in the patient's best interest to continue receiving the treatment, or if Eli Lilly and Company, in consultation with the investigator, decided to discontinue the patient's therapy.

Reference Therapy, Dose, and Mode of Administration: Not applicable.

Variables:

Efficacy:

- Tumor response rate: number of complete responses and partial responses divided by the number of patients qualified for analysis.
- Overall survival: the time from the date of study entry to the date of death from any cause.
- Duration of response: the time from the first objective status assessment of a complete response or a partial response to the time of disease progression or death from any cause.
- Time to progressive disease: the time from study entry to the time of the first observation of disease progression or death from any cause.
- Time to treatment failure: the time from study entry to the time of the first observation of disease progression, death due from any cause, or early discontinuation of treatment.
- Clinical benefit response: change in Karnofsky performance status, patient-reported change in pain intensity, analgesic consumption, and dyspnea.
- Computed tomography scans to objectively determine lung density as an indicator of possible improvement of diaphragmatic mobility and thoracic expansion.

Safety:

- number of units required for transfusions
- adverse event rates
- toxicity rating using the National Cancer Institute Common Toxicity Criteria rating scale
- measurement of vitamin deficiency markers: homocysteine, cystathionine, methylmalonic acid, and methylcitrate (total, I and II).

Health Outcomes:

- Lung Cancer Symptom Scale.

Evaluation Methods:

Statistical:

This was an open-label treatment Phase 2 study with no need for randomization. The primary objective of this clinical trial was to estimate the antitumor activity of pemetrexed in patients with malignant pleural mesothelioma. Up to 61 qualified patients were to be enrolled in a two-stage sequential study with the possibility of stopping the study early for either lack of efficacy or unacceptable toxicity. All confidence intervals for parameters to be estimated were constructed with a significance level of α=0.05.

The primary efficacy endpoint of response rate, which included a 95% confidence interval, was calculated by the formula:

$$\text{Response Rate} = \frac{(\text{CRs} + \text{PRs}) \times 100}{\text{No. of Pts. Qualified for Response Analysis}}$$

The secondary efficacy analyses included the following:

Kaplan-Meier curves for patient overall survival, time to treatment failure, and time to progressive disease, including quartiles for each variable. Kaplan-Meier analyses were done using PROC LIFETEST in Statistical Application Software® (SAS).

Changes from baseline in clinical benefit were defined by the ratio of the number of clinical benefit responders to the number of qualified for benefit analysis:

- Changes from baseline in LCSS item and total scores.
- Changes from baseline in pulmonary function tests.
- Changes from baseline in lung density determinations.

Summary:
During the conduct of this study, Lilly made a programmatic change by requiring supplementation with low-dose folic acid and vitamin B_{12} as a standard component of pemetrexed therapy. This change was made on 10 December 1999 in an effort to improve patient safety. Supplemented patients were defined as those patients who were enrolled in this study and assigned to receive folic acid and vitamin B_{12} on or after 10 December 1999. The nonsupplemented patients were defined as (1) patients who were enrolled in the study before 10 December 1999, and assigned to receive folic acid and vitamin B_{12} from that day onward, and (2) patients who completed study therapy before 10 December 1999 and never received folic acid and vitamin B_{12} for supplementation.

Patients
Seventy patients entered the study; 64 chemonaive patients were enrolled and received at least one dose of the study drug. Forty-three patients were supplemented, and 21 patients were nonsupplemented. The median age of patients in the study was 65 years. Most patients had a diagnosis of epitheloid pleural mesothelioma and had either Stage 3 or Stage 4 disease at the time of enrollment.
Most patients had undergone surgery or received radiotherapy before enrollment. Twenty-one patients had undergone surgery for diagnostic purposes only, 29 patients for palliation, and 1 patient had had undergone surgery with curative intent. The most commonly cited reason for discontinuation of treatment and study withdrawal was lack of efficacy (progressive disease).

Efficacy: Tumor Response Rate
The primary objective of the study was to determine the tumor response rate of patients after treatment with pemetrexed. Sixty-four patients were included in the investigator-determined analysis of tumor response rate and the time-to-event efficacy endpoints.
Table JMDR.1 is a summary tabulation of the investigator assessed tumor response rates for supplemented, nonsupplemented, and all enrolled patients. According to the investigators' assessment, the tumor response rate for all enrolled patients was 14.1% (95% confidence interval [CI], 6.6% to 25.0%).

Table1. Summary of Tumor Response Rate –

Patients	N	CR	PR	Tumor Response Rate (95% CI)
Supplemented	43	0	7	16.3 (6.8-30.7)
Nonsupplemented	21	0	2	9.5 (1.2-30.4)
All enrolled	64	0	9	14.1 (6.6-25.0)

Abbreviations: CI = confidence interval, CR = complete response, PR = partial response.

Table 2 is a detailed summary tabulation of the tumor response rate for the enrolled patients with and without supplementation who were included in the independent assessment. The independent evaluation of tumor response rate included 56 patients. Eight patients were not evaluated because their computed tomography scans were either incomplete or of poor quality. The tumor response rate for enrolled patients according to the independent assessment was 17.9% (95% CI, 8.9% to 30.4%).

Table 2. Summary of Tumor Response Rate – Independent Assessment

Patients	N	CR	PR	Tumor Response Rate (95% CI)
Supplemented	41	0	7	17.1 (7.2-32.1)
Nonsupplemented	15	0	3	20.0 (4.3-48.1)
All enrolled	56	0	10	17.9 (8.9-30.4)

Abbreviations: CI = confidence interval; CR = complete response; N = number of evaluable patients; PR = partial response.

Efficacy: Time-to-Event Endpoints

Table 3 presents a summary of the median overall survival, time to progressive disease, duration of response, and time to treatment failure, with 95% confidence intervals, and the probability of estimates lasting at least 6 months for all enrolled patients (N=64).

Table 3. Summary of Estimates – Time-to-Event Efficacy Endpoints

Time-to-Event Efficacy Endpoint	Median (months) (95% CI)	Probability at 6 months (%)
Overall survival	10.7 (7.7-14.5)	71.5
Time to progressive disease	4.7 (4.2-5.8)	35.9
Duration of response	8.5 (4.4-12.7)	66.7
Time to treatment failure	4.4 (3.1-5.5)	28.1

Abbreviation: CI = confidence interval.

Table 4 provides a summary of median overall survival and time to progressive disease for the supplemented and nonsupplemented patients, respectively.

Table 4. Summary of Estimates – Overall Survival and Time to Progressive Disease

Time-to-Event Efficacy Endpoint	Median (months) (95% CI)	Probability at 6 months (%)
Supplemented Patients (N=43)		
Overall survival	13.0 (8.2, NA)	76.7
Time to progressive disease	4.8 (4.4-6.1)	39.5
Nonsupplemented Patients (N=21)		
Overall survival	8.0 (4.8-14.5)	60.7
Time to progressive disease	3.0 (1.7-5.8)	28.6

Abbreviations: CI = confidence interval; N = number of evaluable patients; NA = not applicable.

Health Outcomes: Clinical Benefit Response

Fifty-six patients were included in the clinical benefit response analysis. Fourteen patients (25.0%) achieved a clinical benefit response on the basis of improvement in self-reported dyspnea, pain intensity, or analgesic consumption, or in clinician-assessed performance status, without concurrent worsening in any of the other measures. Nine of these patients reported improvement in dyspnea, 7 in pain intensity, and 3 in analgesic consumption. Two of these patients had improved performance status.

Mean scores on the LCSS patient scale were relatively unchanged when data were analyzed for all the patients included in the analysis. However, differences were noted when analyses were done for patients according to tumor response. Responders reported improvement in anorexia, fatigue, dyspnea, pain, symptom distress, activity level, global quality of life (QoL), and total LCSS score.

The evaluation of the LCSS observer scale showed that 32% of patients reported improvement in the total LCSS score. Symptoms either improved or remained stable for most patients in all the LCSS measures evaluated.

The small sample size and the large variability in the lung density data did not allow for any specific conclusions to be drawn.

Safety

Sixty-four patients were included in the safety analysis. Enrolled patients completed a median of six cycles of study therapy. Supplemented patients completed a median of six cycles, and nonsupplemented patients a median of two cycles. There were seven dose reductions of pemetrexed (2.1% of doses administered), three among supplemented patients (1.2% of administered doses) and four among nonsupplemented patients (4.3% of administered doses). Neutropenia and febrile neutropenia were the most commonly reported adverse events that led to these reductions. There were 19 dose delays of which only six were of clinical relevance. Five of these delays occurred in supplemented patients and one in a nonsupplemented patient. The clinically important dose delays in supplemented patients were caused by herpes zoster infection (2 patients), myocardial infarction, pain, and asthenia. The single delay among the nonsupplemented patients was attributed to a pleural disorder. Thirteen delays occurred because of scheduling conflicts.

Grade 3 or Grade 4 neutropenia was the most commonly reported laboratory toxicity, reported in 15 patients (23.4%). This included 11 of the 21 nonsupplemented patients (52%). By comparison, only 4 of the 43 supplemented patients (9.4%) reported Grade 3 or Grade 4 neutropenia.

Grade 3 leukopenia was reported in 6 of 21 nonsupplemented (28.6%) and 4 of the 43 supplemented patients (9.4%). Grade 4 leukopenia was reported in 2 nonsupplemented patients (9.5%). There were no reports of Grade 4 leukopenia among supplemented patients.

There were twelve reports of Grade 4 toxicity among nonsupplemented patients. These included eight reports of neutropenia, two reports of leukopenia, one report of thrombocytopenia, and one report of hyperbilirubinemia. There were two reports of Grade 4 toxicity (neutropenia) among the supplemented patients.

Table 5. Summary of Grade 3 and Grade 4 Toxicities
Hematologic Laboratory Data

Laboratory Value	Supplemented Patients (n=43)		Nonsupplemented Patients (n=21)	
	CTC Grade 3 n (%)	CTC Grade 4 n (%)	CTC Grade 3 n (%)	CTC Grade 4 n (%)
Neutrophils	2 (4.7)	2 (4.7)	3 (14.3)	8 (38.1)
Leukocytes	4 (9.3)	0	6 (28.6)	2 (9.5)
Platelets	1 (2.3)	0	0	1 (4.8)
Hemoglobin	1 (2.3)	0	0	0

Fatigue and febrile neutropenia (four reports each) were the most commonly reported clinically important nonlaboratory toxicity. Grade 3 stomatitis was more commonly reported among the nonsupplemented patients, and Grade 3 vomiting was more commonly reported among the supplemented patients. Nausea was reported in 2 supplemented patients and 1 nonsupplemented patient. There was one report of Grade 4 chest pain in a nonsupplemented patient. No Grade 4 toxicity was reported among supplemented patients.
Asthenia was the most commonly reported adverse event. Overall, the five most commonly reported TEAEs were asthenia, nausea, rash, leukopenia, and anorexia. Twenty-three of the 29 patients who reported leukopenia also had neutropenia.
The five most commonly reported TEAEs among supplemented patients were asthenia, nausea, rash, anorexia, and constipation. More TEAEs were reported among nonsupplemented patients and they generally occurred at a higher incidence. The five most commonly reported TEAEs among nonsupplemented patients included asthenia, leukopenia, nausea, rash, and pain.
Twenty-three patients (13 supplemented and 10 nonsupplemented) reported at least one serious adverse event (SAE). Fever was most commonly reported SAE for supplemented patients. Six reports of fever among these supplemented patients included four reports of fever, one report of febrile neutropenia, and one report of fever without neutropenia. Fever (three reports) and leukopenia (three reports) were most commonly reported for nonsupplemented patients. These three reports of fever included two reports of febrile neutropenia and one of fever. The 3 patients with leukopenia also had neutropenia.
Adverse events accounted for discontinuation of treatment and withdrawal from the study in 3 supplemented and 4 nonsupplemented patients. Adverse events that resulted in study withdrawal included elevated creatinine levels, deafness, and arthralgia for the supplemented patients and cerebrovascular accident, dyspnea, abnormal kidney function, and stomatitis for the nonsupplemented patients.
No serious, unexpected, reportable events were reported during the study.
Two patients, 1 supplemented and 1 nonsupplemented, died during the treatment phase of the study and 2 patients within 30 days after administration of the last dose of the study drug. All deaths were attributed to disease progression.
Data obtained for an evaluation of the vitamin deficiency marker status of patients in this study were pooled with data from other studies with pemetrexed. These data were included in an analysis of the relationship between pretherapy homocysteine levels, supplementation with low-dose folic acid and vitamin B_{12}, and hematologic and nonhematologic toxicity encountered in the patients who received treatment with pemetrexed.

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Title	Author	Journal	Citation
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Phase II Study of Pemetrexed With and Without Folic Acid and Vitamin B_{12} as Front-Line Therapy in Malignant Pleural Mesothelioma

By Giorgio V. Scagliotti, Dong-M. Shin, Hedy L. Kindler, Michael J. Vasconcelles, Uwe Keppler, Christian Manegold, Howard Burris, Ulrich Gatzemeier, Johannes Blatter, James T. Symanowski, and James J. Rusthoven

Purpose: This phase II clinical study evaluated the efficacy of pemetrexed for the treatment of malignant pleural mesothelioma (MPM).

Patients and Methods: Patients with a histologically proven diagnosis of MPM, chemotherapy-naive measurable lesions, and adequate organ function received pemetrexed (500 mg/m^2) intravenously over 10 minutes every 3 weeks. After a protocol change, most patients also received folic acid and vitamin B_{12} supplementation to improve safety.

Results: A total of 64 patients were enrolled. Nine (14.1%) of the 64 patients had a partial response. The Kaplan-Meier estimate for median overall survival was 10.7 months. Forty-three patients received vitamin supplementation for all courses of therapy, and 21 patients did not. Seven of the nine responders were vitamin supplemented. The median overall survival was 13.0 months for supplemented patients and 8.0 months for nonsupplemented patients. Vitamin-supplemented patients completed more cycles of therapy than nonsupplemented patients (median, six v two cycles, respectively). Grade 3/4 neutropenia (23.4%) and grade 3/4 leukopenia (18.8%) were the most common laboratory toxicities. Fatigue and febrile neutropenia were the most commonly reported nonlaboratory events (grade 3, 6.3%; grade 4, 0.0% each). The incidence of these toxicities was generally lower in the supplemented patients.

Conclusion: Single-agent pemetrexed for MPM resulted in a moderate response rate (14.1%) and median overall survival of 10.7 months. Patients supplemented with folic acid and vitamin B_{12} tolerated treatment better (less toxicity and more cycles of treatment) and had a 5-month greater median overall survival than nonsupplemented patients. These results indicate that patients with MPM could benefit from single-agent pemetrexed treatment combined with vitamin supplementation.

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MALIGNANT PLEURAL mesothelioma (MPM) is a neoplastic disorder of the pleural lining of the lung, usually presenting at an advanced stage. Because there is no approved or generally accepted standard systemic therapy, patients commonly receive supportive care alone. In this setting, the median survival is reported to be 6 to 18 months (5-year survival, < 5%), and patients usually develop progressive pain and pulmonary compromise as the tumor gradually encases the lung.[1]

Between 50% and 70% of reported cases of MPM are associated with asbestos exposure.[2] There is a long latency period after exposure before the disease emerges (30 to 40 years). There is now rising incidence of MPM throughout much of the world, and it is expected to peak between the years 2010 and 2020.[3,4] In industrialized countries, the incidence of MPM is two per 1,000,000 females and 10 to 30 per 1,000,000 males.[1]

In light of the advanced stage and poor survival of most patients at presentation, numerous cytotoxic agents (single agent or combination) have been evaluated in phase II trials. Response rates from single-agent studies have varied widely, with most ranging from 0% to 15%, with only a few studies reporting higher rates.[5] Median overall survival times from single-agent studies also have varied widely, with most ranging from 7 to 9 months, but a few studies have reported median overall survivals as low as 5 months and as high as 11 months.[6-11]

Pemetrexed (ALIMTA; Eli Lilly and Company, Indianapolis, IN) is a new antifolate with broad antitumor activity. In vitro studies have shown that pemetrexed attacks multiple enzyme targets; namely, dihydrofolate reductase, thymidylate synthase (TS), and glycinamide ribonucleotide formyl transferase. Such targeting contrasts with the single enzyme targets of approved agents such as methotrexate, which acts on dihydrofolate reductase, and fluorouracil and raltitrexed, which inhibit TS.[12-14] Pemetrexed enters the cell primarily through the reduced folate carrier and undergoes extensive intracellular polyglutamation by folylpoly-gamma-glutamate synthetase. Long-term retention of the polyglutamated form of pemetrexed leads to persistently elevated intracellular concentrations and increased cytotoxic potential.[15] Furthermore, polyglutamated pemetrexed has more than 100-fold greater affinity for TS and glycinamide ribonucleotide formyl transferase than the parent compound pemetrexed monoglutamate.[16] This high affinity and long intracellular retention indicates that pemetrexed may be associated with greater clinical activity than other antifolates and TS inhibitors. This multicenter, single-cohort, phase II study was designed to determine the efficacy of pemetrexed as a single agent in chemotherapy-naive patients with advanced MPM.

From the University of Turin, Department of Clinical and Biological Sciences, Torino, Italy; University of Pittsburgh, Pittsburgh, PA; University of Chicago, Chicago, IL; Dana-Farber Cancer Institute, Boston, MA; Lugenfachklinik, Immenhausen; Thorax Klinik, Heidelberg; Krankenhaus Groshansdorf, Groshansdorf; Eli Lilly and Company, Bad Homburg, Germany; Sarah Cannon Cancer Center, Nashville, TN; and Eli Lilly and Company, Indianapolis, IN.

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Address reprint requests to Giorgio V. Scagliotti, MD, University of Turin, Department of Clinical and Biological Sciences, Azienda Ospedaliera S. Luigi, Regione Gonzole 10, 10043 Orbassano (Torino) Italy; email: scagliotti@ihnet.it.

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PATIENTS AND METHODS

Patient Selection

Patients with a histologically proven diagnosis of MPM who were not candidates for curative surgery were eligible for this study. Other major eligibility criteria included presence of bidimensionally and/or unidimensionally measurable lesions by computed tomography (CT) or magnetic resonance imaging; a performance status of ≥ 70 on the Karnofsky scale; an estimated life expectancy of ≥ 12 weeks; adequate bone marrow reserve (absolute neutrophil count [ANC] ≥ 1.5×10^9/L, platelets ≥ 100×10^9/L, and hemoglobin ≥ 9 g/dL); and creatinine clearance (CrCl) ≥ 45 mL/min as calculated by the modified Cockroft and Gault lean body mass formula. Patients with prior systemic chemotherapy were excluded from study, although prior radiation therapy was permitted. Other exclusion criteria were second primary malignancy, documented brain metastases, and inability to interrupt nonsteroidal anti-inflammatory agents.

Study Design

The primary outcome was tumor response. Secondary outcomes included duration of response, survival, time to progressive disease, time to treatment failure, quality of life (QOL), and pulmonary function tests. QOL and pulmonary function test methods and data will be presented in a separate article. This study incorporated a two-stage design[17] to allow for early closure if insufficient clinical activity was evident at the end of the first stage. In the initial design, if at least two of the first 20 assessable patients achieved a response, an additional 21 patients were to be enrolled onto the second stage. Near the end of the first stage, the protocol was amended to require that all patients be supplemented with folic acid and vitamin B_{12} until completion of study therapy in an effort to improve patient safety. In addition, at least 41 patients, rather than 21, were to be enrolled onto the second stage of study, thus ensuring that the number of patients receiving supplementation approximated the original sample size for the entire study. Fully supplemented patients were those who enrolled onto the study on or after December 10, 1999, and who received folic acid and vitamin B_{12} supplementation throughout their treatment cycles. Nonsupplemented patients were those who started receiving study therapy before December 10, 1999, and included patients who completed study therapy without receiving vitamin supplementation and patients who received vitamins at some point after study therapy had begun.

Treatment Regimen

Pemetrexed was supplied as a 40 mg/mL aqueous solution containing 2 mg/mL of the antioxidant monothioglycerol. The starting dose (500 mg/m^2) was diluted in 100 mL of normal saline for intravenous administration over 10 minutes. Drug therapy was repeated at 3-week intervals. The vitamin-supplemented patients received folic acid (350 to 1,000 μg) orally daily beginning 1 to 2 weeks before the first dose of pemetrexed and throughout the study. Vitamin B_{12} (1,000 μg) was given intramuscularly 1 to 2 weeks before the first dose of drug and was administered approximately every 9 weeks throughout study. Dexamethasone (4 mg) or an equivalent corticosteroid was given orally twice daily the day before, day of, and day after each pemetrexed dose to prevent or reduce severity of any skin rash. To reduce risk of delayed renal clearance of pemetrexed, the ingestion of salicylates or nonsteroidal anti-inflammatory agents was not allowed during the 2 days before (5 days for long lasting agents), day of, and 2 days after treatment. Granulocyte colony-stimulating factors were given only to patients who had neutropenic fever, infection with neutropenia, or ANC less than 0.5×10^9/L for at least 5 days. Leucovorin administration was allowed for National Cancer Institute Common Toxicity Criteria (version 2) grade 4 leukopenia or thrombocytopenia or for grade 4 neutropenia lasting more than 5 days.

After each dose of pemetrexed, dose adjustments were made based on platelet and neutrophil nadir counts from the previous cycle of therapy. Once a dose reduction was made, it remained reduced for all subsequent treatments. Patients with ANC less than 0.5×10^9/L received a 25% dose reduction, and patients with platelets less than 50×10^9/L received a 50% dose reduction. If three such dose reductions were required, the patient was discontinued from the study. In addition, if the preceding cycle of therapy resulted in grade 3 or 4 mucositis, the patient received a 50% dose reduction. If diarrhea occurred requiring hospitalization, a 25% dose reduction was made. If grade 3 or 4 nonhematologic effects occurred (with the exception of grade 3 transaminase elevation), the next cycle was delayed until resolution to grade 1 or less. If CrCl decreased to less than 45 mL/min, the next dose was delayed until it improved to ≥ 45 mL/min.

Patient Follow-Up and Measurement of Study End Points

Complete patient history, physical examination, complete blood cell count, calculated CrCl, blood chemistries, and serum vitamin deficiency markers including homocysteine were performed at baseline and before each course of treatment. Complete blood count was performed weekly while patients were on treatment. Tumor response was measured using either conventional CT scan or magnetic resonance imaging scan.

Criteria were established to allow for determination of best tumor response for patients with only bidimensionally measurable disease, only unidimensionally measurable disease, or both. A complete response (CR) was defined as complete disappearance of all measurable and assessable disease with no new lesions, disease-related symptoms, or evidence of nonassessable disease. For patients with only bidimensionally measurable disease, a partial response (PR) was defined as a ≥ 50% reduction from baseline of the sum of products of the perpendicular diameter of target lesions. For those with only unidimensionally measurable disease, a PR was defined as a ≥ 30% decrease in the sum of the greatest diameter of unidimensionally measurable target lesions. For patients with both types of measurable target lesions, one or the other criteria must have been met as indicated above, with no progression in the remaining measurable target lesions. For all response categories, there could be no new lesions, and nonmeasurable lesions must have remained stable or regressed. All objective responses were confirmed approximately 4 weeks after initial documentation. Tumor progression was defined as reappearance of a lesion, appearance of a new lesion/site, a specified degree of progression of existing measurable target lesions, worsening of assessable disease, or death from disease. For patients with only bidimensionally measurable or only unidimensionally measurable disease, progression was considered if the sum of the products of all bidimensionally measurable target disease had increased 50% over the smallest previous sum observed or if there was a 25% increase in the sum of the longest dimension of unidimensionally measurable target lesions over the smallest previous sum observed. For those with both types of lesions, one or the other criteria must have been met. Stable disease was defined at disease that did not qualify for CR, PR, or progression.

Among secondary outcomes, duration of tumor response was defined as the time from first objective status of response to the time of documented disease progression or death from any cause. Overall survival was defined as the time from date of study entry (informed consent date) to date of death from any cause. The time from study entry to the date of last follow-up was used to calculate overall survival for patients alive at the close of the study. Time to progressive disease was defined as time from study entry until time the patient progressed or death from any cause. For patients without a classification of progressive disease, the date of last follow-up was considered right-censored for purposes of these analyses. Time to treatment failure was defined as the time from study entry to the time of first observation of disease progression, death from any cause, or early discontinuation of treatment.

Statistical Methods

The proportion of patients with a PR or CR was calculated for all patients and for the vitamin-supplemented and nonsupplemented subpopulations. Ninety-five percent confidence intervals were calculated based on the F distribution.[18] Survival and other time-to-event end points were analyzed using the Kaplan-Meier method.[19]

RESULTS

From September 1999 to November 2000, 70 patients at 10 centers in Germany, Italy, the United Kingdom, and the United States signed informed consent documents and were assessed for eligibility onto the study. Sixty-four patients met eligibility criteria; the other six patients were considered ineligible to receive study therapy (five patients did not meet enrollment criteria, and one patient withdrew informed consent).

Table 1. Baseline Characteristics for All Enrolled Patients

Characteristic	All Patients (N = 64)		Supplemented Patients (N = 43)		Nonsupplemented Patients (N = 21)	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
Age, years						
Median	65		63		68	
Range	39-80		39-80		54-74	
Sex						
Male	53	82.8	33	76.7	20	95.2
Female	11	17.2	10	23.3	1	4.8
Performance status						
70	7	10.9	5	11.6	2	9.5
80	21	32.8	12	27.9	9	42.9
90	32	50.0	22	51.2	10	47.6
100	4	6.3	4	9.3	0	0
Histologic subtype						
Sarcomatoid	8	12.5	5	11.6	3	14.3
Epithelial	45	70.3	31	72.1	14	66.7
Mixed cell	9	14.1	5	11.6	4	19.0
Unspecified	2	3.1	2	4.7	0	0
Stage at study entry						
IB	4	6.3	3	7.0	1	4.8
II	5	7.8	2	4.7	3	14.3
III	22	34.4	16	37.2	6	28.6
IV	33	51.6	22	51.2	11	52.4

Demographic characteristics of all 64 patients are listed in Table 1. Fully supplemented (n = 43) and nonsupplemented (n = 21) patients are listed separately. Five of the 21 nonsupplemented patients started receiving folic acid and vitamin B_{12} after the start of pemetrexed therapy. As indicated in Table 1, the majority of patients were male, with a median age of 65 years (range, 39 to 80 years). Most patients had a diagnosis of epithelioid pleural mesothelioma at either stage III or IV (International Mesothelioma Interest Group staging system). Metastatic sites included mediastinal and other regional lymph nodes, lung, liver, chest wall, chest, peritoneum, and bone. Two patients (3.1%) had prior radiotherapy, and 30 patients (46.9%) had prior surgery, either palliative (29 patients, 45.3%) or curative (one patient, 1.6%).

All 64 patients received at least one dose of pemetrexed and were included in efficacy and safety analyses. Of the 94 doses intended for the nonsupplemented patients, 90 (95.7%) were delivered at the protocol-defined starting dose, and four (4.3%) were reduced. For fully supplemented patients, 241 doses (98.8%) were delivered at the protocol-defined starting dose, and three (1.2%) were reduced. Thirty-six patients (30 fully supplemented and six nonsupplemented) completed six or more cycles of therapy. Six patients completed only one cycle, and one patient completed 20 cycles. Fully supplemented patients completed more cycles of therapy than the nonsupplemented patients (median, six *v* two cycles; range, one to 20 cycles *v* one to 16 cycles, respectively).

Tumor Response and Time-to-Event Outcomes

Because objective tumor measurements can be difficult in MPM, two separate determinations of best tumor response were performed for each patient. Each investigator made an initial assessment of best response, and an external expert panel independently assessed the best response status of each patient at a later date. The expert panel evaluated 56 patients; eight patients were not evaluated because CT scans submitted to the panel were judged as either incomplete or of poor quality. Tumor response rates calculated from both sets of assessments are listed in Table 2. Among the investigator assessments, no patients experienced a CR; however, nine (14.1%) of the 64 patients had a PR as best tumor response. Among the 43 vitamin-supplemented

Table 2. Response Rates as Determined From Investigator-Determined Best Tumor Response and From Independent Reviewer-Determined Best Tumor Response

Patient Population	Response Rate		No. of Responders		No. of Patients With SD as Best Response	Total No. of Patients
	%	95% CI (%)	CR	PR		
Investigator assessment						
Supplemented	16.3	6.8 to 30.7	0	7	27	43
Nonsupplemented	9.5	1.2 to 30.4	0	2	6	21
All enrolled	14.1	6.6 to 25.0	0	9	33	64
Independent reviewer assessment						
Supplemented	17.1	7.2 to 32.1	0	7	28	41
Nonsupplemented	20.0	4.3 to 48.1	0	3	9	15
All available	17.9	8.9 to 30.4	0	10	37	56

Abbreviations: CI, confidence interval; CR, complete response; PR, partial response; SD, stable disease.

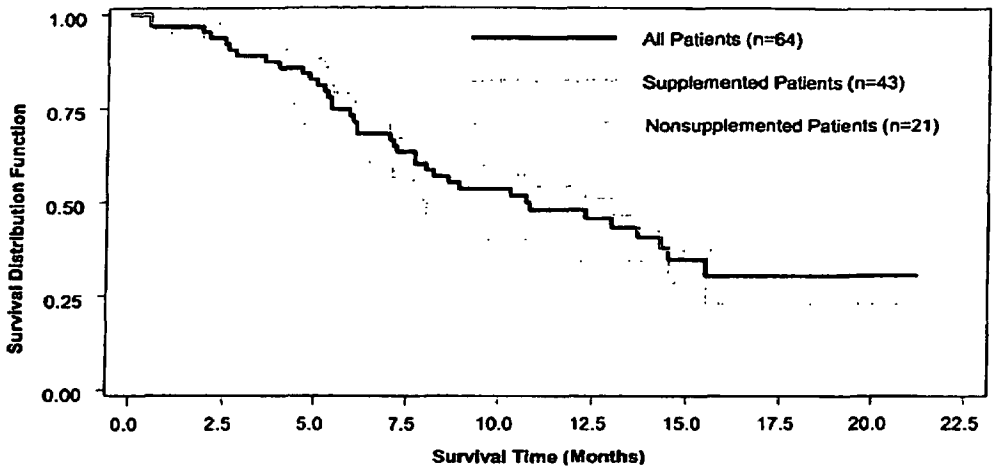

Fig 1. Kaplan-Meier curves of overall survival (months) for all patients and for patients by supplementation status.

patients, seven had a PR, whereas two of the 21 nonsupplemented patients had a PR. In the nonsupplemented group, one of five patients who started receiving supplementation after the start of therapy had a PR. Of the 56 patients included in the independent assessment, 10 patients had a PR as best response (17.9%). Seven responders were among the 41 patients who received vitamin supplementation, whereas the other three responders were among the 15 patients in the nonsupplemented group. All three responding patients in the nonsupplemented group started receiving supplementation after the start of therapy.

The overall survival curves for all enrolled patients and for the supplemented and nonsupplemented subgroups are shown in Fig 1. The median survival for all patients was 10.7 months; supplemented patients had median survival of 13.0 months, compared with 8.0 months for nonsupplemented patients (Table 3). The 6- and 12-month estimates of survival for all patients were 71.5% and 47.8%, respectively. Median time to progressive disease was 4.7 months for all patients, 4.8 months for supplemented patients, and 3.0 months for nonsupplemented patients. The median time to treatment failure for all patients was 4.4 months. Median duration of response among the PR patients (based on investigator assessment) was 8.5 months.

Safety

Neutropenia and febrile neutropenia were the most common reasons for dose reductions; stomatitis and hypokinesia were also reported. Nineteen dose delays were reported; six were clinically relevant (herpes zoster infection, n = 2; myocardial infarction, asthenia, pain, and pleuritis, n = 1 each).

Clinically important laboratory toxicities, as worst common toxicity criteria grade of toxicity, are listed in Table 4. Grade 3/4 neutropenia (23.4%) and grade 3/4 leukopenia (18.8%) were the most common laboratory toxicities. The incidence of grade 3/4 neutropenia among nonsupplemented patients was 52.4% versus only 9.3% for patients who received full vitamin supplementation. Most of the improvement in neutropenia occurred as a reduction in grade 4 severity. Liver function tests revealed elevations in bilirubin, alkaline phosphatase, and alanine transaminase for four patients. These changes were not considered clinically significant with the exception of one patient in the nonsupplemented group who developed grade 4 bilirubinemia.

Nonlaboratory toxicities were relatively infrequent and diverse in nature. Only one grade 4 event was reported (chest pain in a nonsupplemented patient). Twenty-five grade 3 events occurred: 10 events in the 21 nonsupplemented patients and 15 events in the 43 supplemented patients. Fatigue and febrile neutropenia were the most commonly reported events in all patients (6.3% for fatigue and febrile neutropenia each; 4.7% for supplemented patients and 9.5% for nonsupplemented patients for fatigue and febrile neutropenia each), followed by nausea (4.7%), vomiting (3.1%), and stomatitis/pharyngitis (3.1%). Single events (1.6%) included dermatitis or skin desquamation, hand-foot skin reaction, hearing problems, allergic reaction, anorexia, dehydration, diarrhea, dizziness, and genitourinary problems.

There were 23 reports of serious adverse events during the study: 13 in the supplemented patients and 10 in the nonsupplemented patients. Fever (six reports) was the most commonly reported event for supplemented patients, and leukopenia and fever (three reports of each) were the most commonly reported events for nonsupplemented patients. Seven patients had an adverse event that resulted in withdrawal from study; five of these seven patients were nonsupplemented. Reasons for treatment discontinuations included arthralgia and deafness for supplemented patients and cerebrovascular accident, elevated creatinine levels, dyspnea, abnormal kidney function, and stomatitis for nonsupplemented patients.

Table 3. Time-to-Event Outcomes

	Median Time to Event (months)	95% CI (%)
Survival, n = 64	10.7	7.7 to 14.5
Supplemented, n = 43	13.0	8.2 to ∞*
Nonsupplemented, n = 21	8.0	4.8 to 14.5
Time to progression, n = 64	4.7	4.2 to 5.8
Supplemented, n = 43	4.8	4.4 to 6.1
Nonsupplemented, n = 21	3.0	1.7 to 5.8
Time to treatment failure, n = 64	4.4	3.1 to 5.5
Duration of response, investigator-assessed, n = 9	8.5	4.4 to 12.7

Abbreviations: CI, confidence interval.
*Upper limit of CI is ∞ because largest observed death time was within CI.

Table 4. CTC Grade 3 and 4 Laboratory Toxicity by Supplementation Status

	All Patients (N = 64)				Supplemented Patients (N = 43)				Nonsupplemented Patients (N = 21)			
	CTC Grade 3		CTC Grade 4		CTC Grade 3		CTC Grade 4		CTC Grade 3		CTC Grade 4	
Laboratory Value	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Neutrophils	5	7.8	10	15.6	2	4.7	2	4.7	3	14.3	8	38.1
Leukocytes	10	15.6	2	3.1	4	9.3	0	0	6	28.6	2	9.5
Platelets	1	1.6	1	1.6	1	2.3	0	0	0	0	1	4.8
Hemoglobin	1	1.6	0	0	1	2.3	0	0	0	0	0	0
Bilirubin	0	0	1	1.6	0	0	0	0	0	0	1	4.8
Alkaline phosphatase	1	1.6	0	0	1	2.3	0	0	0	0	0	0
AST	0	0	0	0	0	0	0	0	0	0	0	0
ALT	2	3.1	0	0	2	4.7	0	0	0	0	0	0
Creatinine	0	0	0	0	0	0	0	0	0	0	0	0

Abbreviation: CTC, common toxicity criteria (version 2).

Two deaths were reported during study therapy, both during the first cycle of therapy. Both deaths were attributed to disease progression. Two additional deaths occurred within 30 days of administration of the last dose of therapy; these were also attributed to disease progression.

DISCUSSION

Previous single-agent studies in MPM have indicated varying degrees of clinical activity with antifolates such as trimetrexate,[6] edatrexate,[7] and methotrexate.[8] Results of this study show that pemetrexed has moderate antitumor activity in chemotherapy-naive MPM patients. Although the reasons for such antifolate activity in MPM patients are unclear, a recent report has described the presence of a highly expressed, high-affinity alpha folate receptor on mesothelioma cells of all histologic subtypes.[20] Although this type of receptor is only one of several described that can contribute to antifolate transport into cells, the highly expressed presence of such receptors may play a role in the efficient delivery of antifolates, such as pemetrexed, into mesothelioma cells.

The observed (investigator-determined) response rate of 14.1% in this study is comparable with published response rates for single agents.[5-11,21] Furthermore, the relatively large sample size, multicenter nature of this trial, and independent review of patient responses increases confidence that the response rate is a true result for this patient population.

Pemetrexed has been tested in two phase I studies in advanced MPM. In one study of 40 assessable patients, 11 patients had a diagnosis of MPM and received pemetrexed in combination with cisplatin. Five (45.5%) of 11 assessable MPM patients experienced a PR.[22] In the other phase I study, all patients had a diagnosis of MPM, were chemotherapy-naive, and received pemetrexed with carboplatin.[23] Eight (32.0%) of 25 patients assessable for response had a PR.

In our tumor response rate analysis of patient subgroups defined by vitamin supplementation status, data were conflicting; vitamin-supplemented patients had an investigator-assessed response rate higher than that for nonsupplemented patients, whereas the opposite was true for the independent reviewer–assessed response rate. However, sample sizes of these subgroups were small, confidence intervals were overlapping, and CT scans were unassessable among some patients in the reviewer-assessed group. Similarly, data indicated that supplemented patients may have had some improvement in overall survival and time to progressive disease compared with nonsupplemented patients; but again, the number of patients in these subgroups is too small to justify any definitive conclusions. Thus, although patients who received vitamin supplementation were able to receive more pemetrexed, it is not clear that this translated into a true additional benefit from what pemetrexed alone provided. It is also important to note that there was no apparent adverse effect of low-dose folic acid and vitamin B_{12} to pemetrexed therapy on tumor response rate or time-to-event outcomes.

As we have presented previously,[24] patients in this study who responded to therapy also experienced increases in lung volume and motility. These patients also reported improvements in QOL parameters, including dyspnea, pain, symptom distress, and functional capacity. The complete analyses of these data will be presented in a future publication.

The frequency and severity of nonhematologic and nonlaboratory toxicities were low in both vitamin-supplemented and nonsupplemented subgroups. However, supplemented patients had a marked reduction in hematologic toxicity, specifically grade 3/4 neutropenia, as well as a suggested improvement in signs and symptoms of toxicity. Overall improvement in severe toxicity after the addition of low-dose folic acid and B_{12} also has been observed in other pemetrexed studies.[25] Given the favorable safety profile, convenient administration schedule, and moderate single-agent activity, pemetrexed is being investigated as a single agent in other tumor types. Two phase II non–small-cell lung cancer studies of single-agent pemetrexed resulted in response rates of 15.8%[26] and 23.3%.[27] Additional phase II pemetrexed studies in chemotherapy-naive patients with breast,[28] pancreatic,[29] and colorectal[30,31] cancer have yielded response rates ranging from 6% (pancreatic cancer) to 31% (breast cancer).

In summary, pemetrexed demonstrated modest activity as a single agent and was well tolerated, particularly in patients who received low-dose folic acid and vitamin B_{12}. A recently completed phase III study comparing pemetrexed and cisplatin versus cisplatin alone in chemotherapy-naive MPM patients should provide definitive evidence as to whether pemetrexed will become a component of standard therapy in MPM.[32]

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Glossary of Clinical Trials Terms

The following glossary was prepared to help the consumer become familiar with many of the common terms used in clinical trials.

ADVERSE REACTION: (Adverse Event.) An unwanted effect caused by the administration of drugs. Onset may be sudden or develop over time (See **Side Effects**).

ADVOCACY AND SUPPORT GROUPS: Organizations and groups that actively support participants and their families with valuable resources, including self-empowerment and survival tools.

APPROVED DRUGS: In the U.S., the Food and Drug Administration (FDA) must approve a substance as a drug before it can be marketed. The approval process involves several steps including pre-clinical laboratory and animal studies, clinical trials for safety and efficacy, filing of a New Drug Application by the manufacturer of the drug, FDA review of the application, and FDA approval/rejection of application (See **Food and Drug Administration**).

ARM: Any of the treatment groups in a randomized trial. Most randomized trials have two "arms," but some have three "arms," or even more (See **Randomized Trial**).

BASELINE: 1. Information gathered at the beginning of a study from which variations found in the study are measured. 2. A known value or quantity with which an unknown is compared when measured or assessed. 3. The initial time point in a clinical trial, just before a participant starts to receive the experimental treatment which is being tested. At this reference point, measurable values such as CD4 count are recorded. Safety and efficacy of a drug are often determined by monitoring changes from the baseline values.

BIAS: When a point of view prevents impartial judgment on issues relating to the subject of that point of view. In clinical studies, bias is controlled by blinding and randomization (See **Blind** and **Randomization**).

BLIND: A randomized trial is "Blind" if the participant is not told which arm of the trial he is on. A clinical trial is "Blind" if participants are unaware on whether they are in the experimental or control arm of the study; also called masked. (See **Single Blind Study** and **Double Blind Study**).

CLINICAL: Pertaining to or founded on observation and treatment of participants, as distinguished from theoretical or basic science.

CLINICAL ENDPOINT: See **Endpoint**.

CLINICAL INVESTIGATOR: A medical researcher in charge of carrying out a clinical trial's protocol.

CLINICAL TRIAL: A clinical trial is a research study to answer specific questions about vaccines or new therapies or new ways of using known treatments. Clinical trials (also called medical research and research studies) are used to determine whether new drugs or treatments are both safe and effective. Carefully conducted clinical trials are the fastest and safest way to find treatments that work in people. Trials are in four phases: Phase I tests a new drug or treatment in a small group; Phase II expands the study to a larger group of people; Phase III expands the study to an even larger group of people; and Phase IV takes place after the drug or treatment has been licensed and marketed. (See **Phase I**, **II**, **III**, and **IV Trials**).

COHORT: In epidemiology, a group of individuals with some characteristics in common.

COMMUNITY-BASED CLINICAL TRIAL (CBCT): A clinical trial conducted primarily through primary-care physicians rather than academic research facilities.

COMPASSIONATE USE: A method of providing experimental therapeutics prior to final FDA approval for use in humans. This procedure is used with very sick individuals who have no other treatment options. Often, case-by-case approval must be obtained from the FDA for "compassionate use" of a drug or therapy.

COMPLEMENTARY AND ALTERNATIVE THERAPY: Broad range of healing philosophies, approaches, and therapies that Western (conventional) medicine does not commonly use to promote well-being or treat health conditions. Examples include acupuncture, herbs, etc. Internet Address: **http://www.nccam.nih.gov**.

COMPLETED: See **Recruitment Status**

CONFIDENTIALITY REGARDING TRIAL PARTICIPANTS: Refers to maintaining the confidentiality of trial participants including their personal identity and all personal medical information. The trial participants' consent to the use of records for data verification purposes should be obtained prior to the trial and assurance must be given that confidentiality will be maintained.

CONTRAINDICATION: A specific circumstance when the use of certain treatments could be harmful.

CONTROL: A control is the nature of the intervention control.

CONTROL GROUP: The standard by which experimental observations are evaluated. In many clinical trials, one group of patients will be given an experimental drug or treatment, while the control group is given either a standard treatment for the illness or a placebo (See **Placebo** and **Standard Treatment**).

CONTROLLED TRIALS: Control is a standard against which experimental observations may be evaluated. In clinical trials, one group of participants is given an experimental drug, while another group (i.e., the control group) is given either a standard treatment for the disease or a placebo.

DATA SAFETY AND MONITORING BOARD (DSMB): An independent committee, composed of community representatives and clinical research experts, that reviews data while a clinical trial is in progress to ensure that participants are not exposed to undue risk. A DSMB may recommend that a trial be stopped if there are safety concerns or if the trial objectives have been achieved.

DIAGNOSTIC TRIALS: Refers to trials that are are conducted to find better tests or procedures for diagnosing a particular disease or condition. Diagnostic trials usually include people who have signs or symptoms of the disease or condition being studied.

DOSE-RANGING STUDY: A clinical trial in which two or more doses of an agent (such as a drug) are tested against each other to determine which dose works best and is least harmful.

DOUBLE-BLIND STUDY: A clinical trial design in which neither the participating individuals nor the study staff knows which participants are receiving the experimental drug and which are receiving a placebo (or another therapy). Double-blind trials are thought to produce objective results, since the expectations of the doctor and the participant about the experimental drug do not affect the outcome; also called double-masked study. See **Blinded Study**, **Single-Blind Study**, and **Placebo**.

DOUBLE-MASKED STUDY: See **Double-Blind Study**.

DRUG-DRUG INTERACTION: A modification of the effect of a drug when administered with another drug. The effect may be an increase or a decrease in the action of either substance, or it may be an adverse effect that is not normally associated with either drug.

DSMB: See **Data Safety and Monitoring Board**

EFFICACY: (Of a drug or treatment). The maximum ability of a drug or treatment to produce a result regardless of dosage. A drug passes efficacy trials if it is effective at the dose tested and against the illness for which it is prescribed. In the procedure mandated by the FDA, Phase II clinical trials gauge efficacy, and Phase III trials confirm it (See **Food and Drug Administration (FDA)**, **Phase II** and **III Trials**).

ELIGIBILITY CRITERIA: Summary criteria for participant selection; includes Inclusion and Exclusion criteria. (See **Inclusion/Exclusion Criteria**)

EMPIRICAL: Based on experimental data, not on a theory.

ENDPOINT: Overall outcome that the protocol is designed to evaluate. Common endpoints are severe toxicity, disease progression, or death.

ENROLLING: The act of signing up participants into a study. Generally this process involves evaluating a participant with respect to the eligibility criteria of the study and going through the **informed consent** process.

EPIDEMIOLOGY: The branch of medical science that deals with the study of incidence and distribution and control of a disease in a population.

EXCLUSION/INCLUSION CRITERIA: See **Inclusion/Exclusion Criteria**

EXPANDED ACCESS: Refers to any of the FDA procedures, such as compassionate use, parallel track, and treatment IND that distribute experimental drugs to participants who are failing on currently available treatments for their condition and also are unable to participate in ongoing clinical trials.

EXPERIMENTAL DRUG: A drug that is not FDA licensed for use in humans, or as a treatment for a particular condition (See **Off-Label Use**).

FDA: See **Food and Drug Administration**

FOOD AND DRUG ADMINISTRATION (FDA): The U.S. Department of Health and Human Services agency responsible for ensuring the safety and effectiveness of all drugs, biologics, vaccines, and medical devices, including those used in the diagnosis, treatment, and prevention of HIV infection, AIDS, and AIDS-related opportunistic infections. The FDA also works with the blood banking industry to safeguard the nation's blood supply. Internet address: **http://www.fda.gov/**.

HYPOTHESIS: A supposition or assumption advanced as a basis for reasoning or argument, or as a guide to experimental investigation.

INCLUSION/EXCLUSION CRITERIA: The medical or social standards determining whether a person may or may not be allowed to enter a clinical trial. These criteria are based on such factors as age, gender, the type and stage of a disease, previous treatment history, and other medical conditions. It is important to note that inclusion and exclusion criteria are not used to reject people personally, but rather to identify appropriate participants and keep them safe.

IND: See **Investigational New Drug**

INFORMED CONSENT: The process of learning the key facts about a clinical trial before deciding whether or not to participate. It is also a continuing process throughout the study to provide information for participants. To help someone decide whether or not to participate, the doctors and nurses involved in the trial explain the details of the study.

INFORMED CONSENT DOCUMENT: A document that describes the rights of the study participants, and includes details about the study, such as its purpose, duration, required procedures, and key contacts. Risks and potential benefits are explained in the informed consent document. The participant then decides whether or not to sign the document. Informed consent is not a contract, and the participant may withdraw from the trial at any time.

INSTITUTIONAL REVIEW BOARD (IRB): 1. A committee of physicians, statisticians, researchers, community advocates, and others that ensures that a clinical trial is ethical and that the rights of study participants are protected. All clinical trials in the U.S. must be approved by an IRB before they begin. 2. Every institution that conducts or supports biomedical or behavioral research involving human participants must, by federal regulation, have an IRB that initially approves and periodically reviews the research in order to protect the rights of human participants.

INTENT TO TREAT: Analysis of clinical trial results that includes all data from participants in the groups to which they were randomized (See **Randomization**) even if they never received the treatment.

INTERVENTION NAME: The generic name of the precise intervention being studied.

INTERVENTIONS: Primary interventions being studied: types of interventions are Drug, Gene Transfer, Vaccine, Behavior, Device, or Procedure.

INVESTIGATIONAL NEW DRUG: A new drug, antibiotic drug, or biological drug that is used in a clinical investigation. It also includes a biological product used *in vitro* for diagnostic purposes.

IRB: See **Institutional Review Board**

MASKED: The knowledge of intervention assignment. See **Blind**

NATURAL HISTORY STUDY: Study of the natural development of something (such as an organism or a disease) over a period of time.

NEW DRUG APPLICATION (NDA): An application submitted by the manufacturer of a drug to the FDA - after clinical trials have been completed - for a license to market the drug for a specified indication.

OFF-LABEL USE: A drug prescribed for conditions other than those approved by the FDA.

OPEN-LABEL TRIAL: A clinical trial in which doctors and participants know which drug or vaccine is being administered.

ORPHAN DRUGS: An FDA category that refers to medications used to treat diseases and conditions that occur rarely. There is little financial incentive for the pharmaceutical industry to develop medications for these diseases or conditions. Orphan drug status, however, gives a manufacturer specific financial incentives to develop and provide such medications.

PEER REVIEW: Review of a clinical trial by experts chosen by the study sponsor. These experts review the trials for scientific merit, participant safety, and ethical considerations.

PHARMACOKINETICS: The processes (in a living organism) of absorption, distribution, metabolism, and excretion of a drug or vaccine.

PHASE I TRIALS: Initial studies to determine the metabolism and pharmacologic actions of drugs in humans, the side effects associated with increasing doses, and to gain early evidence of effectiveness; may include healthy participants and/or patients.

PHASE II TRIALS: Controlled clinical studies conducted to evaluate the effectiveness of the drug for a particular indication or indications in patients with the disease or condition under study and to determine the common short-term side effects and risks.

PHASE III TRIALS: Expanded controlled and uncontrolled trials after preliminary evidence suggesting effectiveness of the drug has been obtained, and are intended to gather additional information to evaluate the overall benefit-risk relationship of the drug and provide and adequate basis for physician labeling.

PHASE IV TRIALS: Post-marketing studies to delineate additional information including the drug's risks, benefits, and optimal use.

PLACEBO: A placebo is an inactive pill, liquid, or powder that has no treatment value. In clinical trials, experimental treatments are often compared with placebos to assess the treatment's effectiveness. (See **Placebo Controlled Study**).

PLACEBO CONTROLLED STUDY: A method of investigation of drugs in which an inactive substance (the placebo) is given to one group of participants, while the drug being tested is given to another group. The results obtained in the two groups are then compared to see if the investigational treatment is more effective in treating the condition.

PLACEBO EFFECT: A physical or emotional change, occurring after a substance is taken or administered, that is not the result of any special property of the substance. The change may be beneficial, reflecting the expectations of the participant and, often, the expectations of the person giving the substance.

PRECLINICAL: Refers to the testing of experimental drugs in the test tube or in animals - the testing that occurs before trials in humans may be carried out.

PREVENTION TRIALS: Refers to trials to find better ways to prevent disease in people who have never had the disease or to prevent a disease from returning. These approaches may include medicines, vaccines, vitamins, minerals, or lifestyle changes.

PROTOCOL: A study plan on which all clinical trials are based. The plan is carefully designed to safeguard the health of the participants as well as answer specific research questions. A protocol describes what types of people may participate in the trial; the schedule of tests, procedures, medications, and dosages; and the length of the study. While in a clinical trial, participants following a protocol are seen regularly by the research staff to monitor their health and to determine the safety and effectiveness of their treatment (See **Inclusion/Exclusion Criteria**).

QUALITY OF LIFE TRIALS (or Supportive Care trials): Refers to trials that explore ways to improve comfort and quality of life for individuals with a chronic illness.

RANDOMIZATION: A method based on chance by which study participants are assigned to a treatment group. Randomization minimizes the differences among groups by equally distributing people with particular characteristics among all the trial arms. The researchers do not know which treatment is better. From what is known at the time, any one of the treatments chosen could be of benefit to the participant (See **Arm**).

RANDOMIZED TRIAL: A study in which participants are randomly (i.e., by chance) assigned to one of two or more treatment arms of a clinical trial. Occasionally placebos are utilized. (See **Arm** and **Placebo**).

RECRUITING: The period during which a trial is attempting to identify and enroll participants. Recruitment activites can include advertising and other ways of solicting interest from possible particpants. (See **recruitment status** and **enrolling**).

RECRUITMENT STATUS: Indicates the current stage of a trial, whether it is planned, ongoing, or completed. Possible values include:

- Not yet recruiting: participants are not yet being recruited or enrolled
- Recruiting: participants are currently being recruited and enrolled
- Enrolling by invitation: participants are being (or will be) selected from a predetermined population
- Active, not recruiting: study is ongoing (i.e., patients are being treated or examined), but enrollment has completed
- Completed: the study has concluded normally; participants are no longer being examined or treated (i.e., last patient's last visit has occurred)
- Suspended: recruiting or enrolling participants has halted prematurely but potentially will resume
- Terminated: recruiting or enrolling participants has halted prematurely and will not resume; participants are no longer being examined or treated
- Withdrawn: study halted prematurely, prior to enrollment of first participant

RISK-BENEFIT RATIO: The risk to individual participants versus the potential benefits. The risk/benefit ratio may differ depending on the condition being treated.

SCREENING TRIALS: Refers to trials which test the best way to detect certain diseases or health conditions.

SIDE EFFECTS: Any undesired actions or effects of a drug or treatment. Negative or adverse effects may include headache, nausea, hair loss, skin irritation, or other physical problems. Experimental drugs must be evaluated for both immediate and long-term side effects (See **Adverse Reaction**).

SINGLE-BLIND STUDY: A study in which one party, either the investigator or participant, is unaware of what medication the participant is taking; also called single-masked study. (See **Blind** and **Double-Blind Study**).

SINGLE-MASKED STUDY: See **Single-Blind Study.**

STANDARD TREATMENT: A treatment currently in wide use and approved by the FDA, considered to be effective in the treatment of a specific disease or condition.

STANDARDS OF CARE: Treatment regimen or medical management based on state of the art participant care.

STATISTICAL SIGNIFICANCE: The probability that an event or difference occurred by chance alone. In clinical trials, the level of statistical significance depends on the number of participants studied and the observations made, as well as the magnitude of differences observed.

STUDY ENDPOINT: A primary or secondary outcome used to judge the effectiveness of a treatment.

STUDY TYPE: The primary investigative techniques used in an observational protocol; types are Purpose, Duration, Selection, and Timing.

SUSPENDED: See **Recruitment Status**

TERMINATED: See **Recruitment Status**

TOXICITY: An adverse effect produced by a drug that is detrimental to the participant's health. The level of toxicity associated with a drug will vary depending on the condition which the drug is used to treat.

TREATMENT IND: IND stands for Investigational New Drug application, which is part of the process to get approval from the FDA for marketing a new prescription drug in the U.S. It makes promising new drugs available to desperately ill participants as early in the drug development process as possible. Treatment INDs are made available to participants before general marketing begins, typically during Phase III studies. To be considered for a treatment IND a participant cannot be eligible to be in the definitive clinical trial.

TREATMENT TRIALS: Refers to trials which test new treatments, new combinations of drugs, or new approaches to surgery or radiation therapy.

WITHDRAWN: See **Recruitment Status**

Glossary Sources:

AIDSinfo: **Glossary of HIV/AIDS-Related terms 4th Edition**.
CenterWatch, Inc. Patient Resources: Glossary.
ECRI (formerly the Emergency Care Research Institute).
Eli Lilly and Company: Lilly Clinical Trials Glossary.
MediStudy.com Inc: ClinicalTrials: A-Z Glossary.
National Cancer Institute: **Cancer.gov Dictionary.**

Background Information

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of a new drug for which a marketing application has not been approved; (iii) the drug is not being commercially promoted or advertised; and (iv) the sponsor of the drug is actively pursuing marketing approval with due diligence. FDA must be notified in writing in advance of commencing any such charges, in an information amendment submitted under §312.31. Authorization for charging goes into effect automatically 30 days after receipt by FDA of the information amendment, unless the sponsor is notified to the contrary.

(3) *Noncommercialization of investigational drug.* Under this section, the sponsor may not commercialize an investigational drug by charging a price larger than that necessary to recover costs of manufacture, research, development, and handling of the investigational drug.

(4) *Withdrawal of authorization.* Authorization to charge for an investigational drug under this section may be withdrawn by FDA if the agency finds that the conditions underlying the authorization are no longer satisfied.

(Collection of information requirements approved by the Office of Management and Budget under control number 0910-0014)

[52 FR 8831, Mar. 19, 1987, as amended at 52 FR 19476, May 22, 1987]

§312.10 Waivers.

(a) A sponsor may request FDA to waive applicable requirement under this part. A waiver request may be submitted either in an IND or in an information amendment to an IND. In an emergency, a request may be made by telephone or other rapid communication means. A waiver request is required to contain at least one of the following:

(1) An explanation why the sponsor's compliance with the requirement is unnecessary or cannot be achieved;

(2) A description of an alternative submission or course of action that satisfies the purpose of the requirement; or

(3) Other information justifying a waiver.

(b) FDA may grant a waiver if it finds that the sponsor's noncompliance would not pose a significant and unreasonable risk to human subjects of the investigation and that one of the following is met:

(1) The sponsor's compliance with the requirement is unnecessary for the agency to evaluate the application, or compliance cannot be achieved;

(2) The sponsor's proposed alternative satisfies the requirement; or

(3) The applicant's submission otherwise justifies a waiver.

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[52 FR 8831, Mar. 19, 1987, as amended at 52 FR 23031, June 17, 1987]

Subpart B—Investigational New Drug Application (IND)

§312.20 Requirement for an IND.

(a) A sponsor shall submit an IND to FDA if the sponsor intends to conduct a clinical investigation with an investigational new drug that is subject to §312.2(a).

(b) A sponsor shall not begin a clinical investigation subject to §312.2(a) until the investigation is subject to an IND which is in effect in accordance with §312.40.

(c) A sponsor shall submit a separate IND for any clinical investigation involving an exception from informed consent under §50.24 of this chapter. Such a clinical investigation is not permitted to proceed without the prior written authorization from FDA. FDA shall provide such written authorization 30 days after FDA receives the IND or earlier.

[52 FR 8831, Mar. 19, 1987, as amended at 61 FR 51529, Oct. 2, 1996]

§312.21 Phases of an investigation.

An IND may be submitted for one or more phases of an investigation. The clinical investigation of a previously untested drug is generally divided into three phases. Although in general the phases are conducted sequentially, they may overlap. These three phases of an investigation are a follows:

(a) *Phase 1.* (1) Phase 1 includes the initial introduction of an investigational new drug into humans. Phase 1 studies are typically closely monitored and may be conducted in patients or

normal volunteer subjects. These studies are designed to determine the metabolism and pharmacologic actions of the drug in humans, the side effects associated with increasing doses, and, if possible, to gain early evidence on effectiveness. During Phase 1, sufficient information about the drug's pharmacokinetics and pharmacological effects should be obtained to permit the design of well-controlled, scientifically valid, Phase 2 studies. The total number of subjects and patients included in Phase 1 studies varies with the drug, but is generally in the range of 20 to 80.

(2) Phase 1 studies also include studies of drug metabolism, structure-activity relationships, and mechanism of action in humans, as well as studies in which investigational drugs are used as research tools to explore biological phenomena or disease processes.

(b) *Phase 2.* Phase 2 includes the controlled clinical studies conducted to evaluate the effectiveness of the drug for a particular indication or indications in patients with the disease or condition under study and to determine the common short-term side effects and risks associated with the drug. Phase 2 studies are typically well controlled, closely monitored, and conducted in a relatively small number of patients, usually involving no more than several hundred subjects.

(c) *Phase 3.* Phase 3 studies are expanded controlled and uncontrolled trials. They are performed after preliminary evidence suggesting effectiveness of the drug has been obtained, and are intended to gather the additional information about effectiveness and safety that is needed to evaluate the overall benefit-risk relationship of the drug and to provide an adequate basis for physician labeling. Phase 3 studies usually include from several hundred to several thousand subjects.

§312.22 General principles of the IND submission.

(a) FDA's primary objectives in reviewing an IND are, in all phases of the investigation, to assure the safety and rights of subjects, and, in Phase 2 and 3, to help assure that the quality of the scientific evaluation of drugs is adequate to permit an evaluation of the drug's effectiveness and safety. Therefore, although FDA's review of Phase 1 submissions will focus on assessing the safety of Phase 1 investigations, FDA's review of Phases 2 and 3 submissions will also include an assessment of the scientific quality of the clinical investigations and the likelihood that the investigations will yield data capable of meeting statutory standards for marketing approval.

(b) The amount of information on a particular drug that must be submitted in an IND to assure the accomplishment of the objectives described in paragraph (a) of this section depends upon such factors as the novelty of the drug, the extent to which it has been studied previously, the known or suspected risks, and the developmental phase of the drug.

(c) The central focus of the initial IND submission should be on the general investigational plan and the protocols for specific human studies. Subsequent amendments to the IND that contain new or revised protocols should build logically on previous submissions and should be supported by additional information, including the results of animal toxicology studies or other human studies as appropriate. Annual reports to the IND should serve as the focus for reporting the status of studies being conducted under the IND and should update the general investigational plan for the coming year.

(d) The IND format set forth in §312.23 should be followed routinely by sponsors in the interest of fostering an efficient review of applications. Sponsors are expected to exercise considerable discretion, however, regarding the content of information submitted in each section, depending upon the kind of drug being studied and the nature of the available information. Section 312.23 outlines the information needed for a commercially sponsored IND for a new molecular entity. A sponsor-investigator who uses, as a research tool, an investigational new drug that is already subject to a manufacturer's IND or marketing application should follow the same general format, but ordinarily may, if authorized by the manufacturer, refer to the manufacturer's

Metabolism at a Glance

J.G. Salway BSc, MSc, PhD
Senior Lecturer in Medical Biochemistry
School of Biological Sciences
University of Surrey
Guildford GU2 5XH

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Blackwell Science Ltd
Editorial Offices:
Osney Mead, Oxford OX2 0EL
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Amino acid metabolism, folate metabolism, and the '1-carbon pool', part 1: purine biosynthesis

D8a

23

Chart 23 opposite. Purine biosynthesis

The '1-carbon pool'

This term describes the 1-carbon residues associated with *S*-adenosylmethionine and folate which are available for metabolic reactions.

S-Adenosylmethionine (SAM)

SAM, which is formed from methionine, is the major donor of methyl groups for biosynthetic reactions. It can, for example, methylate noradrenaline to form adrenaline, as shown in the chart opposite. Other important reactions involving SAM include the methylation of phosphatidylethanolamine to phosphatidylcholine, and the formation of creatine.

The folate '1-carbon' units

The vitamin folate is reduced in two stages by dihydrofolate reductase to produce the active form, tetrahydrofolate (THF). THF is a versatile carrier of 1-carbon units in the following oxidation states: methyl, methylene, methenyl, and formyl. These THF compounds, which are interconvertible, together with SAM, comprise what is known as the '1-carbon' pool.

Amino acids and the '1-carbon' pool

Serine is converted to glycine, in a reaction catalysed by serine hydroxymethyl transferase, with the transfer of a methyl group to the THF so as to form N^5,N^{10}-methylene THF. This reaction is particularly important in the thymidylate synthase reaction described in Chapter 24. Oxidation of glycine in mitochondria by the glycine cleavage enzyme also produces N^5,N^{10}-methylene THF (see Chapter 19).

Tryptophan is oxidized to *N*-formylkynurenine which, in the presence of formamidase, yields kynurenine and the toxic product formate. THF accepts the formate, producing N^{10}-formyl THF.

Methionine, as mentioned above, is the precursor of SAM which, following transfer of the methyl group, forms homocysteine. Methionine can be regenerated from homocysteine by methylation using N^5-methyl THF in a salvage pathway. NB: This reaction, catalysed by homocysteine methyltransferase, requires vitamin B_{12} and lack of this vitamin can lead to folate being caught in the 'methyl-folate' trap (see below).

Amino acid metabolism and purine synthesis

Glycine contributes the C-4, C-5, and N-7 atoms to the purine ring in a reaction catalysed by glycinamide ribonucleotide (GAR) synthetase (see Chart 23).

Aspartate is an important donor of nitrogen atoms during purine biosynthesis, contributing the N-1 atom to the purine ring, and the $-NH_2$ group in the adenylosuccinate synthetase reaction of the pathway which forms AMP from inosine monophosphate (IMP) (see Diagram 23.1).

Glutamine plays a very important role in nucleotide metabolism. It donates the nitrogen atoms which form N-9 and N-3 of the purine ring. It also participates in the amination of xanthine monophosphate (XMP) to form guanosine monophosphate (GMP) (Diagram 23.1).

Biosynthesis of purines

Purine nucleotides can be synthesized *de novo*. They can also be reclaimed from existing nucleosides by the so-called 'salvage pathway' (see Chapter 24). The *de novo* pathway needs '1-carbon' units from the folate pool, and several amino acids as detailed below.

De novo pathway for purine biosynthesis

The pathway starts with ribose 5-phosphate formed by the pentose phosphate pathway (see Chart 23). This is activated to form phosphoribosyl pyrophosphate (PRPP). A total of 11 reactions is needed to form IMP (inosine monophosphate or inosinic acid), which is the precursor of the adenine- and guanine-containing nucleotides. The important roles of glutamine and aspartate as amino donors are emphasized. A total of three glutamine molecules and one aspartate molecule is needed for the synthesis of GMP. Similarly, a total of two glutamine and two aspartate molecules is needed for AMP synthesis. A molecule of glycine is needed in each case.

The *de novo* pathway is controlled by feedback inhibition of PRPP amidotransferase by AMP and GMP. In primary gout this feedback control is impaired, causing increased production of purines resulting in the increased formation of their sparingly soluble excretory product, urate.

Vitamin B_{12} and the 'methyl-folate trap'

Vitamin B_{12}, or more precisely its methyl cobalamin derivative, is an essential coenzyme for the transfer of methyl groups in the methionine salvage pathway (see Chart 23). Accordingly, in B_{12} deficiency, THF cannot be released and remains trapped as N^5-methyl THF. Eventually, according to the hypothesis, all the body's folate becomes trapped in the N^5-methyl THF form, and so folate deficiency develops secondary to B_{12} deficiency. Because blood cells turn over rapidly, they need nucleotides for nucleic acid synthesis and are vulnerable to folate deficiency, which causes megaloblastic anaemia.

The methyl-folate trap hypothesis explains the observation that, although the haematological symptoms of B_{12} deficiency respond to folate treatment, the neurological degeneration progresses. Remember that the other enzyme for which B_{12} is a coenzyme is methylmalonyl CoA mutase (see Chapters 18 and 19). Accumulation of methylmalonyl CoA may interfere with the biosynthesis of lipids needed for the myelin sheath.

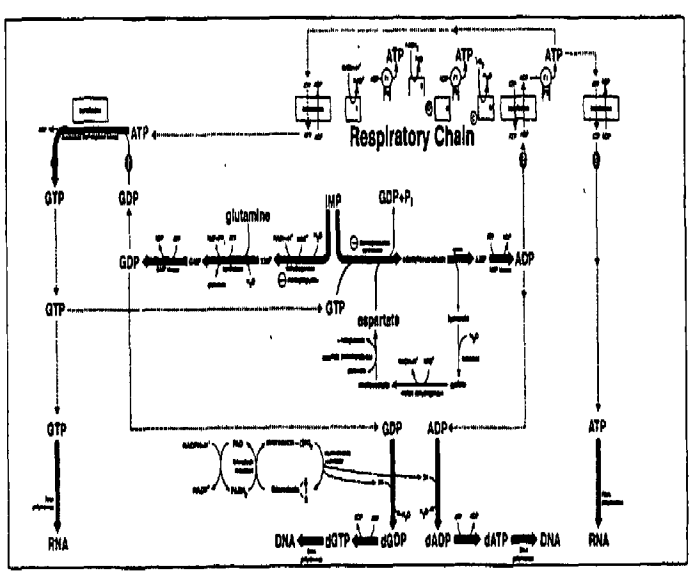
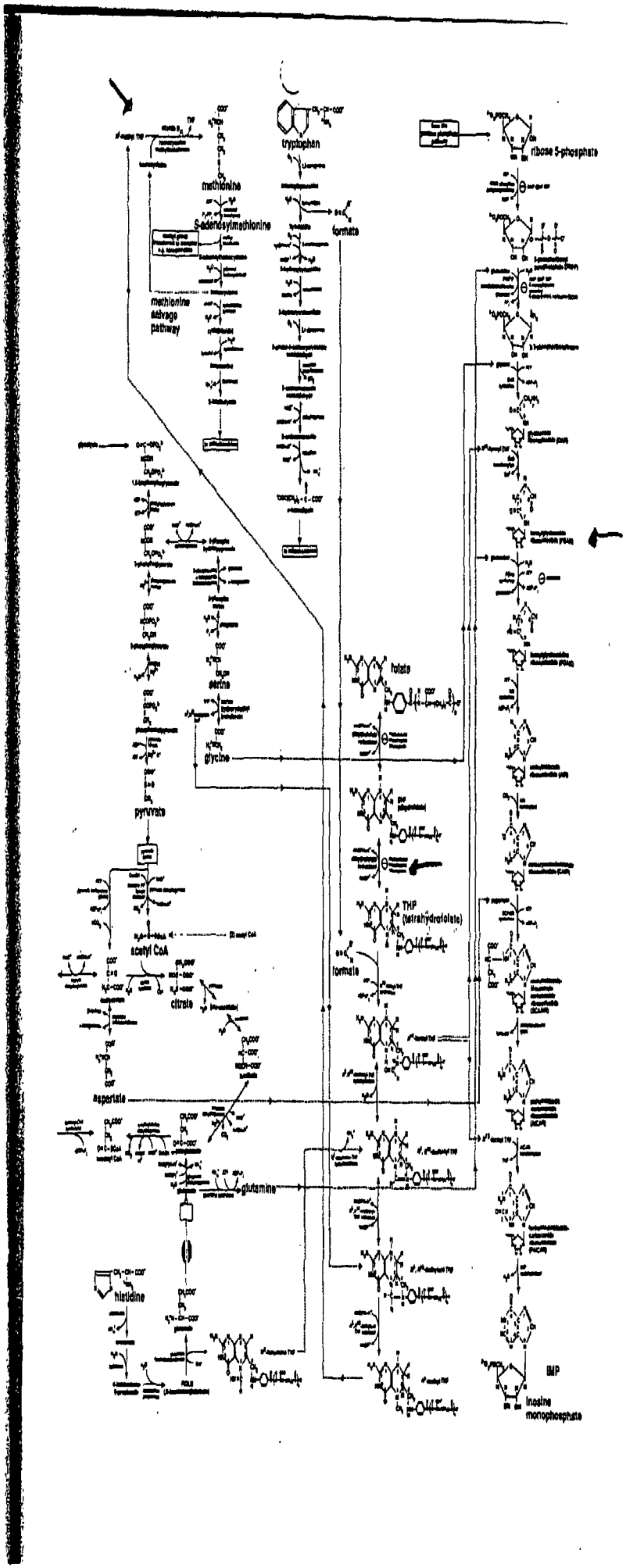


Diagram 23.1. Conversion of IMP to ATP. IMP reacts with aspartate in the presence of GTP to form adenylsuccinate, which is cleaved to form fumarate and AMP. The AMP can be phosphorylated to ADP, which undergoes oxidative phosphorylation to form ATP.

Conversion of IMP to GTP. IMP is oxidized to xanthine monophosphate (XMP), which is aminated to form GMP, which is phosphorylated to form GDP. GDP is phosphorylated by ATP in a reaction catalysed by nucleoside diphosphate kinase. Alternatively, when the Krebs cycle is active, GTP is formed from GDP by succinyl CoA synthetase.

Formation of dATP (deoxyadenosine triphosphate) and dGTP (deoxyguanosine triphosphate). The deoxyribonucleosides dATP and dGTP are formed by first reducing ADP and GDP to dADP and dGDP in the presence of ribonucleotide reductase. These are subsequently phosphorylated to form dATP and dGTP, which can be used for the synthesis of DNA.

Table A



Amino acid metabolism, folate metabolism, and the '1-carbon pool', part II: pyrimidine biosynthesis

D8b

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Chart 24 opposite. Biosynthesis of pyrimidines.

Amino acid metabolism and pyrimidine biosynthesis

The pyrimidine ring is derived from glutamine, aspartate and bicarbonate. The first reaction occurs in the cytosol and produces carbamoyl phosphate from bicarbonate, glutamine and two molecules of ATP. This is similar to the mitochondrial reaction involved in the urea cycle, which differs in that it forms carbamoyl phosphate from bicarbonate and NH_4^+ ions. The rest of the pyrimidine ring is donated by aspartate and, after ring closure and oxidation, orotate is formed. It is at this stage that phosphoribosyl pyrophosphate (PRPP) is added to yield orotidine monophosphate (OMP) which, following decarboxylation, produces uridine monophosphate (UMP), which is the common precursor of the pyrimidine-containing nucleotides.

Conversion of UMP to UTP and CTP

UMP is phosphorylated by a specific UMP kinase to form uridine diphosphate (UDP), which in turn is phosphorylated by the non-specific nucleoside diphosphate kinase to yield uridine triphosphate (UTP). When UTP is aminated, cytidine triphosphate (CTP) is formed.

Formation of deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP)

dCTP is formed from CDP by ribonucleoside reductase, as described for the production of the purine-containing deoxyribonucleotides in Chapter 23.

The pathway for the formation of dTTP is quite distinct from that used to produce dATP, dGTP and dCTP. The pathway starts with dCDP, which is dephosphorylated and deaminated to yield deoxyuridine monophosphate (dUMP). This is methylated by N^5,N^{10}-methylene THF which is oxidized to dihydrofolate (DHF) in the reaction catalysed by thymidylate synthase, and deoxythymidine monophosphate (dTMP) is formed. The dTMP is now phosphorylated by dTMP kinase and nucleoside diphosphate kinase to produce dTTP.

Let us return to the DHF, which is formed by the thymidylate synthase reaction. This is reduced by dihydrofolate reductase, which regenerates tetrahydrofolate (THF). The cycle is completed when this THF participates in the serine hydroxymethyltransferase reaction which produces glycine and N^5,N^{10}-methylene THF, which is now available once more for the thymidylate synthase reaction.

Cancer chemotherapy

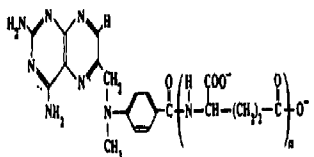
Because rapidly dividing cancer cells have a great demand for DNA synthesis, much attention has been directed at the pathways for nucleotide synthesis as the target for chemotherapeutic intervention. These drugs are classified by pharmacologists as 'antimetabolites' and fall into the following categories: glutamine antagonists, folate antagonists, antipyrimidines and antipurines.

Glutamine antagonists

The importance of glutamine for the biosynthesis of purines and pyrimidines has been emphasized already. Azaserine and diazo-oxo-norleucine (DON) irreversibly inhibit the enzymes involved in the glutamine-utilizing reactions (see Chart 23), and reduce the supply of DNA available to cancer cells.

Folate antagonists

Methotrexate, which is a close structural analogue of folate, inhibits DHF reductase. This prevents the reduction of DHF to THF, as shown in the chart opposite. Consequently, in the absence of THF, serine hydroxymethyltransferase is unable to generate the N^5,N^{10}-methylene-THF needed by thymidylate synthase for dTMP production.



methotrexate

The clinical benefit to patients treated with high doses of methotrexate is improved by the use of folinic acid, N^5-formyl THF (also known as leucovorin), which 'rescues' normal cells from the toxic effects of methotrexate.

Antipyrimidines

Fluorouracil inhibits thymidylate synthase and thus prevents the conversion of dUMP to dTMP.

Antipurines

Mercaptopurine inhibits purine biosynthesis at several stages. It inhibits PRPP-amidotransferase (see Chart 23), IMP dehydrogenase and adenylosuccinate synthetase (see Diagram 23.1).

Salvage pathways for the recycling of purines and pyrimidines

When nucleic acids and nucleotides are degraded, the free purine and pyrimidine bases are formed. These can be recycled by 'salvage pathways' which require much less ATP compared with the energy-intensive *de novo* pathways. The salvage pathways require specific phosphoribosyl transferases (PRTs) whose functions are analogous to that of orotate PRT (see chart opposite).

AMP salvage

$$\text{adenine} + \text{PRPP} \xrightarrow[\text{adenine PRT}]{} \text{AMP} + PP_i$$

IMP and GMP salvage

Both hypoxanthine and guanine can be used as substrates by the enzyme involved:

$$\text{hypoxanthine} + \text{PRPP} \xrightarrow[\text{hypoxanthine-guanine PRT}]{} \text{IMP} + PP_i$$

$$\text{guanine} + \text{PRPP} \xrightarrow[\text{hypoxanthine-guanine PRT}]{} \text{GMP} + PP_i$$

UMP and TMP salvage

$$\text{uracil} + \text{PRPP} \xrightarrow[\text{uracil-thymine PRT}]{} \text{UMP} + PP_i$$

$$\text{thymine} + \text{PRPP} \xrightarrow[\text{uracil-thymine PRT}]{} \text{TMP} + PP_i$$

NB: Uracil-thymine PRT cannot use cytosine as a substrate.

Lesch-Nyhan syndrome

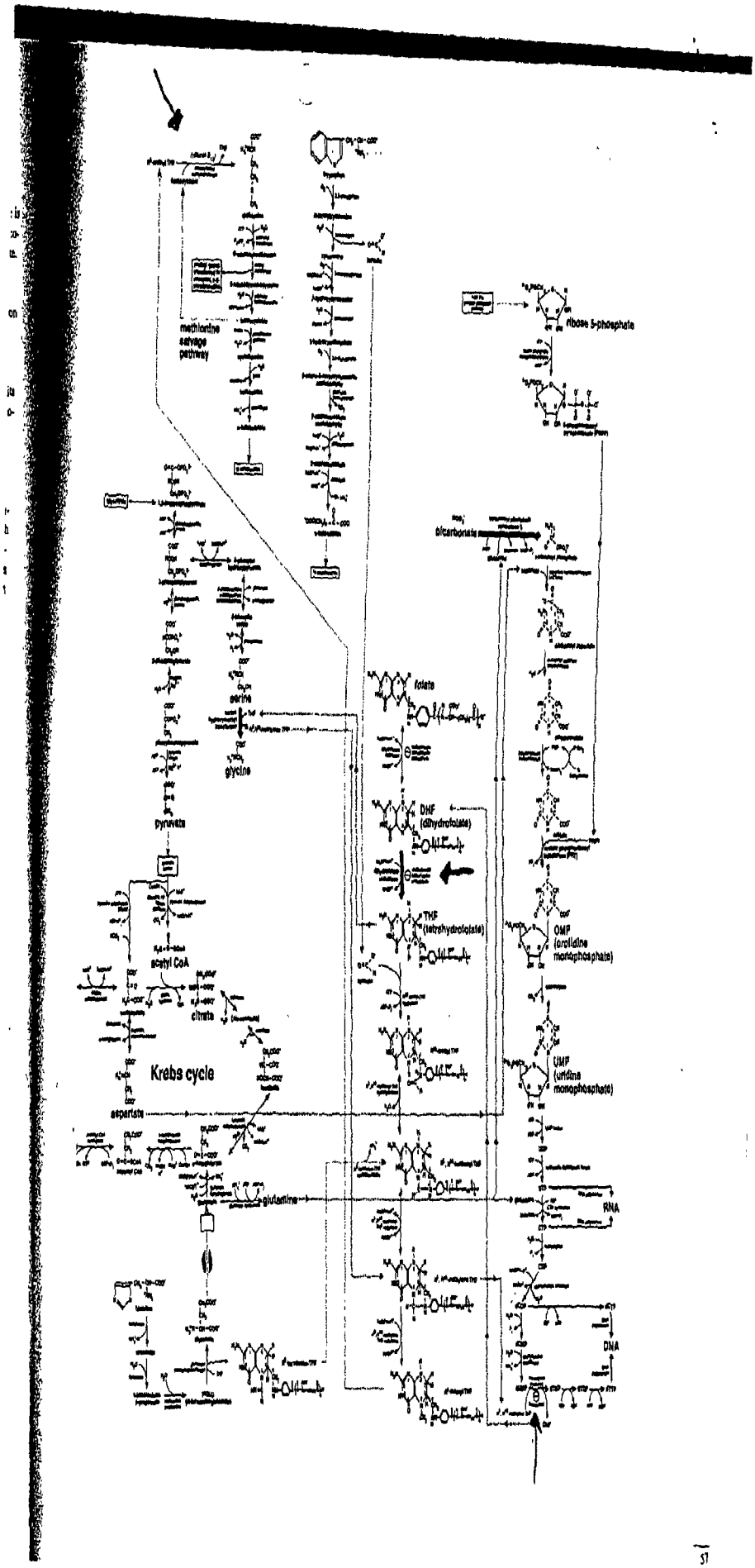
This is an extremely rare disorder caused by almost total deficiency of hypoxanthine-guanine PRT. In this condition, which is characterized by severe self-mutilation, the salvage pathway is inactive. Consequently, the free purines hypoxanthine and guanine, are instead oxidized by xanthine oxidase to urate.

The antiviral drug AZT (azidothymidine)

AZT is an analogue of thymidine which can be phosphorylated to form the nucleotide triphosphate, azidothymidine triphosphate (AZTTP).

AZTTP inhibits the viral DNA-polymerase which is an RNA-dependent polymerase. The host cell's DNA-dependent polymerase is relatively insensitive to inhibition by AZTTP.

Table B



*2138

PROPHYLACTIC MASTECTOMY (PM) AND OOPHORECTOMY (PO) IN WOMEN UNDERGOING BRCA1/2 TESTING. *D. Schrag, K.J. Kalkbrenner, T.L. Light, K.A. Schneider, J.E. Garber. Dana Farber Cancer Institute, Boston; MA.*

Women tested for BRCA1/2 mutations may consider PM and/or PO based on the results of genetic testing for predisposing mutations. A cohort of 88 women with at least 10% risk of inherited breast/ovarian cancer provided information about attitudes towards PM and PO before testing and again at mean 5.5 months following results disclosure. 46 women had prior breast/ovarian cancer (CA); 42 women had not had cancer (NC). Before genetic testing, 8 women had had PM, 12 PO and 5 therapeutic oophorectomy. At baseline, 37/80 had discussed PM with a physician and 33/71 had discussed PO 8/80 were considering PM and 24/71 PO. Following BRCA disclosure, 6 women underwent PM (3 CA, 3 NC) and 5 had PO (2 CA, 3 NC); one woman (NC) had both procedures. Mutations were identified in all women having prophylactic surgery following results disclosure except for 2 who had PM with indeterminate results but abnormal breast biopsies. In addition, 13 were still considering PM (8+, 4?) and 19 were considering PO (12+, 6?, 1−) For the entire cohort, no cancers have been detected at PM; one borderline ovarian cancer was found at PO. PM and PO are often considered by women who have BRCA1/2 mutation testing even with indeterminate test results.

*2139

LY231514 (MTA): RELATIONSHIP OF VITAMIN METABOLITE PROFILE TO TOXICITY. *C. Niyikiza, J. Walling, D. Thornton, D. Seitz, and R. Allen. Eli Lilly and Company, Indianapolis, IN, and Univ of Colorado Health Science Center, Denver, CO.*

LY231514 (MTA) is a new generation multitargeted antifolate antimetabolite with inhibitory activity against thymidylate synthase, dihydrofolate reductase and glycinamide ribonucleotide formyl transferase. Of a total of 246 patients (pts) in phase II trials treated with MTA (600 mg/m² IV over 10 minutes once every 21 days) 118 pts also had vitamin metabolites measured. Because earlier studies with other antifolates had suggested that nutritional status may play a role in the likelihood that a patient will experience severe toxicity, levels of the vitamin metabolites homocysteine, cystathionine and methylmalonic acid were measured at baseline and once each cycle thereafter. A multivariate statistical analysis of the data was conducted in order to determine which among a set of pre-specified predictors (creatinine clearance, albumin levels, liver enzyme levels, and vitamin metabolites) might correlate with toxicity. There was a strong correlation between baseline homocysteine levels and the development of the following toxicities at any time during the study: CTC Grade 4 neutropenia (57 pts, $p < 0.0001$), Grade 4 thrombocytopenia (13 pts, $p < 0.0001$), Grade 3 or 4 mucositis (8 pts, $p < 0.0003$), and Grade 3 or 4 diarrhea (8 pts, $p < 0.004$). Cystathionine levels did not correlate with hematologic toxicity or mucositis but were moderately correlated with fatigue ($p < 0.04$). Maximum cystathionine levels doubled from baseline during treatment with MTA. No correlation between toxicity (CTC Grades as defined above) and the remaining pre-specified predictors was seen. Toxicity was seen in all patients with homocysteine levels above a threshold concentration of 10 μM. A correlation over time between homocysteine levels and CTC Grade 4 neutropenia and thrombocytopenia and CTC Grade 3 or 4 mucositis was also observed, but only in the first two cycles of treatment. Maximum homocysteine levels did not appear to change from baseline during treatment with MTA.

*2140

PHASE I CHEMOPREVENTION CLINICAL TRIAL OF CURCUMIN *A.L. Cheng, J.K. Lin, M.M. Hsu, T.S. Shen, J.Y. Ko, J.T. Lin, B.J. Lin, M.S. Wu, H.S. Yu, S.H. Jee, G.S. Chen, T.M. Chen, C.A. Chen, M.K. Lai, Y.S. Pu, M.H. Pan, Y.J. Wang, C.C. Tsai, C.Y. Hsieh. National Taiwan University College of Medicine, Taipei, Taiwan; and Kaohsiung Medical College, Kaohsiung, Taiwan.*

Curcumin (diferuloylmethane), a yellow substance from the root of the plant Curcuma longa Linn., has been demonstrated to inhibit murine carcinogenesis of skin, stomach, intestine and oral cavity. A phase-I clinical trial was conducted to examine the toxicology, the pharmacokinetics and the biologically effective dose of curcumin in humans. Five types of high-risk individual were eligible: 1. recently-resected urinary bladder cancer (BC), 2. arsenic Bowen's disease (BD), 3. uterine cervical intraepithelial neoplasia (CIN), 4. oral leukoplakia (OL), and 5. intestinal metaplasia of gastric mucosa (IM). The starting dose was 500 mg/day, taken orally for 3 months. If no any ≥ Grade II toxicity was noted in at least 3 p'ts, the dose was escalated successively to 1000 (level II), 2000 (level III), 4000 (level IV), and 8000 mg/day (level V). Lesion sites were biopsied before and 3 months after taking curcumin. Serum curcumin was quantitated by HPLC method. In a total of 25 p'ts enrolled, no treatment-related toxicity was noted up to 8000 mg/day (level V). Serum concentration usually peaked at 1 to 2 hours after oral intake, and gradually declined within 12 hours. The average peak serum concentrations after taking 4000 mg, 6000 mg and 8000 mg of curcumin were 0.41 ± 0.07 μM, 0.57 ± 0.05 μM, and 1.75 ± 0.80 μM, respectively. Although 3 of 25 p'ts proceeded to develop frank malignancies, histological improvement of the precancerous lesions was seen in 1 (level III) of the 2 p'ts with BC, 2 (both level IV) of 7 p'ts with OL, 1 (level II) of 6 p'ts with IM, 1 (level I) of 4 p'ts with CIN, and 2 (level I and III) of 6 p'ts with BD. Although curcumin is probably non-toxic even up to more than 8000 mg/day, the bulky volume of drug tablets became a limiting factor itself. Therefore, for future phase II studies, doses close to 8000 mg/day may be recommended.

*2141

FACTORS INFLUENCING THE DECISION TO UNDERGO BRCA1/2 GENE TESTING: A STUDY OF ASHKENAZI JEWISH WOMEN WITH A PERSONAL HISTORY OF BREAST CANCER (BC), ENROLLED IN AN ONTARIO CANCER GENETICS NETWORK PROTOCOL. *K.A. Phillips, J. Hunter, E. Warner, W. Meschino, G. Glendon, I.L. Andrulis and P.J. Goodwin. Mt Sinai Hospital, Princess Margaret Hospital, Toronto-Sunnybrook Regional Cancer Center, North York General Hospital, Toronto, Ontario, Canada.*

The purpose of this study was to examine the contribution of demographic, medical, psychosocial, and cultural/religious factors in decision making regarding testing for BRCA1 and BRCA2 mutations, in Canadian Jewish women with BC, unselected for family history. A self-administered questionnaire was developed and distributed, (after genetic counseling), to 134 individuals enrolled in a research-based testing program for Ashkenazi women. Data for the first 52 participants are presented. The response rate was 40 (77%). Respondents had the following demographic features: age 40–75 years (median = 59), married 83%, had children 92%, post-secondary education 55%, practicing Jew 88%, extra health insurance 77%, median age of BC diagnosis = 50. No patient had ovarian cancer (OC). 45% had at least one 1st degree relative with BC or OC (median perceived risk for being a gene carrier 50%). 35% had no affected relatives (median perceived risk for being a carrier = 15%). The 5 factors most frequently identified as "definitely an important factor in my decision making" were, desire to contribute to research (90%), curiosity (77%), potential benefit to other family members (64%), potential for personal cancer prevention (59%), and impact on ovarian cancer screening practice (41%). 53% and 38% of women respectively, identified a potential change in their perspective on prophylactic oophorectomy and mastectomy as at least "somewhat important." Main concerns related to insurance discrimination (35%); confidentiality (30%), accuracy and interpretability of results (33%), potential impact on marriage prospects for family members (20%), and focus on the Jewish community (15%). Potential employer discrimination and impact on life planning were "not a factor" for most (90%,82%). The focus on factors unrelated to personal physical health is notable. The generalisability of these results to women not affected by BC requires further study. Final results for the 134 patients will be presented.

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A PHASE I AND PHARMACOKINETIC (PK) STUDY OF THE MULTITARGETED ANTIFOL (MTA) LY231514 WITH FOLIC ACID (Meeting abstract).

Sub-category: Other
Category: Clinical Pharmacology
Meeting: 1998 ASCO Annual Meeting

Abstract No: 866

Author(s): L Hammond, M Villalona-Calero, SG Eckhardt, R Drengler, C Aylesworth, T Johnson, M Hidalgo, G Rodriguez, S Diab, P Monroe, D Thornton, Hoff D Vo, E Rowinsky

Abstract: MTA (LY 231514) is a new antifol that inhibits multiple folate-dependent enzymes, including thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyl transferase. Initial phase I trials demonstrated major antitumor responses when MTA was given as a 10 min I.V. infusion, however, myelosuppression precluded dose escalation above 500-600 mg/m2. Since preclinical studies indicated that folic acid supplementation increases the eherapeutic index of MTA, the feasibility of administering folic acid 5 mg daily for 5 days starting 2 days before MTA in minimally- and heavily-pretreated pts was evaluated to determine if folic acid supplementation ameliorates the toxif effects of MTA, permitting significant dose-escalation above the recommended phase II dose of MTA alone. Thus far, 21 pts with solid cancers have received 55 courses at the following dose levels: 600, 700, and 800 mg/m2. Drug-related toxicities have included neutropenia, anemia, and thrombocytopenia, which have been more severe in heavily-pretreated pts. Other toxicities (grade 1-2) include rash, somnolence, fatigue, leg edema, and diminished renal function manifested by a decrease in creatinine clearance. One pt taking a non-steroidal anti-inflammatory agent experienced severe toxicities at the 800 mg/m2 dose, which resolved after administration of leucovorin and thymidine. One partial response in a pt with metastatic colon cancer has been observed. PK and vitamin (folic acid) metabolite profiles were done during cycles 1 and 3 at 600 to 800 mg/m2. To date, serum folic acid levels do not appear to be related to toxicity, but homocysteine was significantly elevated in the pt with severe toxicities at the 800 mg/m2 dose. Thus far, heavily- and minimally-pretreated patients have tolerated MTA at 600 and 800 mg/m2 and accrual continues at 700 and 900 mg/m2, respectively. These results indicate that folic acid supplementation appears to permit MTA dose escalation.

Other Abstracts in this Sub-Category

1. **PHARMACOKINETICS OF IRINOTECAN AND ITS ACTIVE METABOLITE SN-38 IN CHILDREN WITH RECURRENT SOLID TUMORS AFTER PROTRACTED LOW DOSE IV IRINOTECAN (Meeting abstract).**
   Meeting: 1998 ASCO Annual Meeting Abstract No: 715 First Author: Stewart C
   Category: Clinical Pharmacology - Other
2. **POPULATION PHARMACOKINETIC (PK) MODEL FOR TOPOTECAN (TPT) (Meeting abstract).**
   Meeting: 1998 ASCO Annual Meeting Abstract No: 716 First Author: PB Laub
   Category: Clinical Pharmacology - Other
3. **CYCLOSPORIN A (CsA) STRONGLY ENHANCES ORAL BIOAVAILABILITY OF PACLITAXEL (pac) IN CANCER PATIENTS (Meeting abstract).**
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   Category: Clinical Pharmacology - Other

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Abstracts by L Hammond

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   Meeting: 2001 ASCO Annual Meeting Abstract No: 368 First Author: C H Takimoto
   Category: Clinical Pharmacology - Phase I Trials
2. **SB-408075, a Tumor-Activated Prodrug Maytansinoid Immunoconjugate Directed to the C242 Antigen: a Phase I, Pharmacokinetic and Biologic Correlative Study.**
   Meeting: 2001 ASCO Annual Meeting Abstract No: 273 First Author: Anthony William Tolcher
   Category: Clinical Pharmacology - Phase I Trials
3. **A PHASE I AND PHARMACOKINETIC (PK) STUDY OF THE FARNESYLTRANSFERASE INHIBITOR, R115777 IN COMBINATION WITH GEMCITABINE (Gem).**
   Meeting: 2000 ASCO Annual Meeting Abstract No: 5A First Author: Amita Patnaik
   Category: Clinical Pharmacology

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***Journal of Clinical Oncology* Articles by L Hammond**

Folic Acid Supplementation Prevents Deficient Blood Folate Levels and Hyperhomocysteinemia During Longterm, Low Dose Methotrexate Therapy for Rheumatoid Arthritis: Implications for Cardiovascular Disease Prevention

SARAH L. MORGAN, JOSEPH E. BAGGOTT, JEANNETTE Y. LEE, and GRACIELA S. ALARCÓN

ABSTRACT. Objective. To determine the effect of longterm methotrexate (MTX) therapy and folic acid supplementation on folate nutriture and homocysteine levels in patients with rheumatoid arthritis.

Methods. A double blind, placebo controlled trial lasting one year was conducted at one academic medical center. A total of 79 patients taking low dose MTX were followed up to one year. The patients were randomized to receive placebo or 5 or 27.5 mg folic acid supplementation per week.

Results. Plasma and erythrocyte folate levels and plasma homocysteine levels were determined. The folate nutriture of patients taking low dose MTX declined without folic acid supplementation. Plasma homocysteine levels increased significantly over a one year period in the placebo group. Low folate nutriture and hyperhomocysteinemia occurred with greater frequency in the placebo group than in the folic acid supplemented groups.

Conclusion. For longterm, low dose MTX therapy, there are now at least 3 reasons to consider supplementation with folic acid (a low cost prescription): (1) to prevent MTX toxicity, (2) to prevent or treat folate deficiency, and (3) to prevent hyperhomocysteinemia, considered by many investigators to be a risk factor for cardiovascular disease. (J Rheumatol 1998;25:441–6)

Key Indexing Terms:
HOMOCYSTEINE METHOTREXATE FOLIC ACID RHEUMATOID ARTHRITIS

Methotrexate (MTX) is an antifolate widely used in low doses in the therapy of autoimmune diseases, psoriasis, inflammatory bowel disease, and asthma[1–6]. It is now the leading disease modifying antirheumatic drug for the treatment of rheumatoid arthritis (RA), a disease that affects roughly 1% of the adult population around the world[7–1]. About 184,000 patients with RA were treated with MTX in 1995 (Wyeth-Ayerst, personal communication) in the United States alone. We have shown that the resulting interference with folate metabolism is correlated with toxicity and that folic acid (pteroylglutamic acid), in doses of 5–27.5 mg/week, lowers the toxicity of low dose MTX therapy for RA[7,8]. We also postulate that folate mediated processes are involved in mechanisms of efficacy and toxicity of MTX[9]. Homocystsine is a sulfur-containing amino acid that may be remethylated via a folate dependent reaction to form methionine. Folate nutriture is a major determinant of plasma homocysteine levels and blood folate levels are generally inversely correlated to homocysteine levels[10–16]. Plasma homocysteine levels > 15 µmol/l have been implicated as an independent risk factor for cardiovascular disease[17–27].

Because of these relationships, monitoring of blood folates (plasma and erythrocyte) and homocysteine levels during longterm MTX therapy may have important clinical implications. We report plasma and erythrocyte (red blood cells, RBC) folates and plasma homocysteine levels during a one year randomized, double blind, placebo controlled trial to study the effect of folic acid supplementation during low dose MTX therapy for RA[8]. We hypothesized that chronically low folate nutriture and chronic hyperhomocysteinemia would be more frequent in the non-folic acid supplemented group.

From the Division of Clinical Nutrition and Dietetics and the Division of Nutritional Biochemistry, Department of Nutrition Sciences, Schools of Medicine, Health Related Professions, and Dentistry, Division of Hematology/Oncology and Division of Clinical Immunology and Rheumatology, Department of Medicine, the University of Alabama at Birmingham, Birmingham, Alabama, USA.

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S.L. Morgan, MD, RD, Associate Professor; J.E. Baggott, PhD, Assistant Professor, Division of Nutritional Biochemistry, Department of Nutrition Sciences, Schools of Medicine, Health Related Professions, and Dentistry; J.Y. Lee, PhD, Senior Biostatistician, Division of Hematology and Oncology, Department of Medicine; G.S. Alarcón, MD, MPH, Professor and Associate Director, Multipurpose Arthritis and Musculoskeletal Disease Center, Division of Clinical Immunology and Rheumatology, Department of Medicine.

Address reprint requests to Dr. S.L. Morgan, 256A Webb, 1675 University Boulevard, Birmingham, AL 35294-3360. E-Mail: slmorgan@uab.edu

Submitted April 24, 1997; revision accepted September 18, 1997.

MATERIALS AND METHODS

Participants and study design. The study was approved by the Institutional Review Board of the University of Alabama. As described[8], the participants were 79 patients aged 19–78 years of age who fulfilled the American College of Rheumatology revised criteria for RA[28]. The primary outcomes of that study were the effect of folic acid supplementation on measures of toxicity and efficacy[8]. Patients were enrolled in a one year, double blind, placebo controlled trial to evaluate the effects of 5 mg (low folic acid (FA) group) or 27.5 mg (high FA group) folic acid supplementation per week during low dose MTX therapy[8]. The patients took folic acid or identical placebo capsules on the 5 days of the week when MTX was not ingested. MTX was taken in a single dose on one day of the week. There were no significant differences between the 3 groups regarding mean age, sex, racial distribution, mean disease duration, previous use of folic acid containing vitamins, IgM rheumatoid factor positivity defined as > 30 IU/ml or > 1:160 titer, concurrent use of aspirin or nonsteroidal antiinflammatory drugs, mean prednisone dose, or cumulative MTX dose at the end of the trial. The mean (± standard deviation) age of the entire population was 53.2 (± 13.6) years. Seventy-six percent of the patients were women. Seventy-six percent of the patients were Caucasian. Sixty-two percent were concurrently taking prednisone and 80% were IgM rheumatoid factor positive. If patients had abnormal values on a vitamin panel min A, plasma and RBC folate, vitamin B_{12}, vitamin B_6, thiamine/riboflavin, and vitamin C) other than folates, the abnormality was treated with appropriate single vitamin supplementation. No other vitamin supplements were permitted except as noted.

Patients were examined immediately before MTX initiation (Visit 1) and after a mean of 13, 26, 39, and 53 weeks of therapy (Visits 2–5, respectively) for clinical evaluation and venipuncture. A one day dietary recall using the Minnesota Nutrition Data System software, Food Database version 6A, Nutrient Database version F21, was performed at each visit to assess nutrient intakes[29].

Vitamin and homocysteine assays. At Visit 1, blood was drawn for the assessment of vitamins B_6 and vitamin B_{12} nutriture[30,31]. Blood for plasma and RBC folate levels was drawn 5–7 days after MTX dosing in a tube containing EDTA and assayed at all visits using a MTX resistant *Lactobacillus casei* microbiological assay[32,33]. The blood was drawn 5–7 days after MTX dosing. Criteria for adequacy of folate status were based on the categories of folate adequacy established by Selhub and Rosenberg[34]. Serum folate levels were considered to be low when values were < 6.7 nmol/l and RBC folate levels were considered low when values were < 315 nmol/l[34]. Homocysteine levels were assayed using high performance liquid chromatography[35]. Values > 15 µmol/l were considered to be elevated[36]. Blood was generally processed within 30 min of phlebotomy and plasma frozen at −70°C until time of analysis.

Statistical analysis. Two way repeated measures analyses of variance were used to evaluate the effects of treatment group, time, and its interaction on plasma and RBC folate levels, and on plasma homocysteine levels after baseline. If a significant treatment effect was detected, Tukey's studentized range test was used to evaluate pairwise comparisons between treatment groups[37].

Fisher's exact test was used to compare the 3 treatment groups with respect to the proportions of patients who had 0, 1, or more than 1 occurrence of deficient plasma or RBC folate levels or elevated plasma homocysteine levels (see Tables 1 and 2).

Linear regression analyses were performed to evaluate the change in homocysteine levels over time after baseline in each treatment group.

Pearson correlation analyses were done to evaluate the baseline relationship of RBC and plasma folate levels with plasma homocysteine levels and the correlation of dietary folate, vitamin B_{12}, and vitamin B_6 intakes with plasma homocysteine levels.

RESULTS

Plasma and RBC folate levels during the trial. Figures 1 and 2 show plasma and RBC folate levels at each visit. There were no significant differences between plasma and RBC folate levels at baseline ($p > 0.05$). There were striking differences between treatment groups with respect to plasma and RBC folate levels across followup visits. For plasma and RBC folate levels, significant differences ($p < 0.001$) were found for all 3 pairwise comparisons (low FA vs high FA, high FA vs placebo, and low FA vs placebo) at followup visits. Significant treatment–visit interactions were observed for plasma folate ($p < 0.001$) and RBC folate ($p = 0.003$).

Multiple instances of a low blood folate level are likely to be more clinically significant than an isolated low blood folate level. The number of patients with 0, 1, or more than

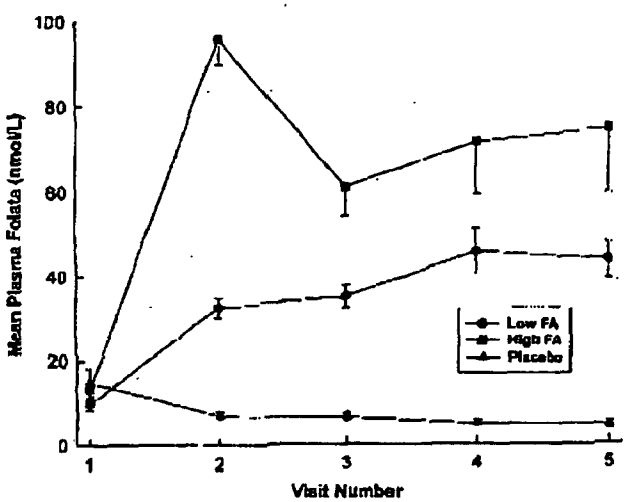

Figure 1. Plasma folate levels (nmol/l) in the Low FA, High FA, and Placebo groups during one year of MTX therapy. Visits are 3 months apart.

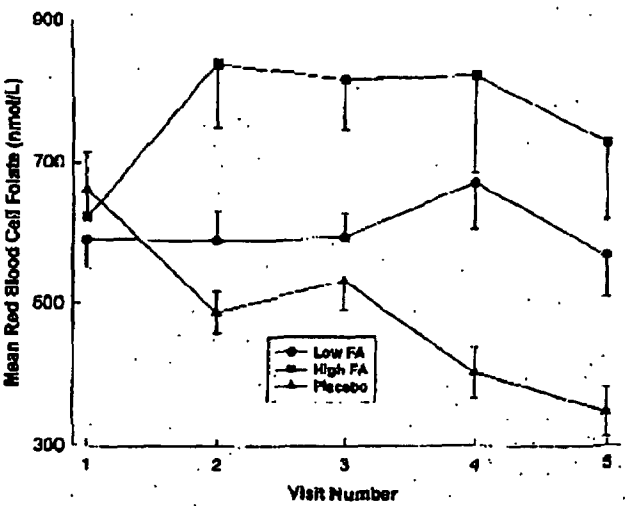

Figure 2. RBC folate levels (nmol/l) in the Low FA, High FA, and Placebo groups during one year of MTX therapy. Visits are 3 months apart.

l low blood folate level during Visits 2–5 is shown in Table 1. A clear majority of the patients in the placebo group had 2 or more deficient levels, while the vast majority in the folic acid supplemented groups maintained normal blood folate levels throughout the study ($p < 0.001$).

Plasma homocysteine levels during the trial. There were no significant differences in baseline plasma homocysteine levels between groups at Visit 1 ($p > 0.05$). Normal mean homocysteine levels in 40–70-year-old women and men range from 8.0 to 10.3 μmol/l[20,38]. The mean values in the placebo group were in the range of 13.6–21.7 μmol/l at Visits 2–5 (Figure 3), substantially above the range of normal means. After baseline, for Visits 2–5, the mean overall plasma homocysteine level was 17.4 μmol/l in the placebo group. The means in the folic acid supplemented groups at almost all visits were comparable with the above population norms[20,38].

Figure 3 shows plasma homocysteine levels over time. At Visit 1, significant treatment differences were observed with respect to homocysteine levels. The low FA and high FA groups differed significantly from the placebo groups across Visits 2–5 ($p < 0.001$).

Multiple instances of hyperhomocysteinemia are more likely to be significant than an isolated elevated homocysteine level. The percentage of patients with more than one

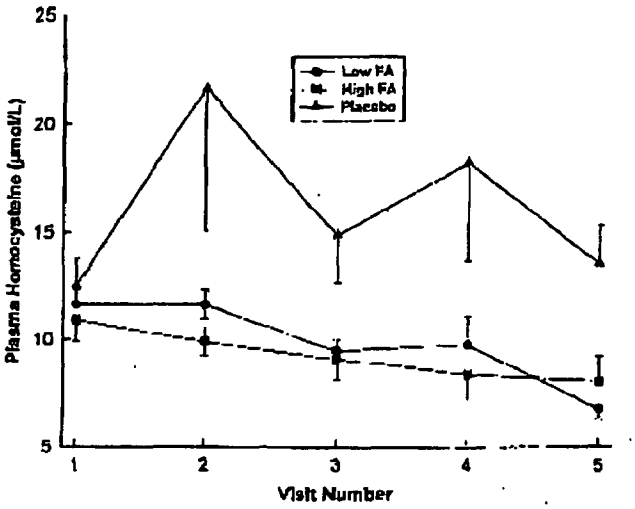

Figure 3. Plasma homocysteine (μmol/l) levels in the Low FA, High FA, and Placebo groups during one year of MTX therapy. Visits are 3 months apart.

elevated plasma homocysteine level, assayed every 3 months, is shown in Table 2. About 40% of the placebo group had 2 or more elevated plasma homocysteine levels during the course of the trial. In contrast, chronic hyperho-

Table 1. Chronic deficient folate nutriture in MTX treated patients with RA receiving high and low dose folic acid supplements and placebo.

	Number (%) of Patients with 0, 1, 2, 3, or 4 Deficient Plasma or RBC Folate Assays During Followup Visits				
	0	1	2	3	4
Group					
Low FA	19 (76)	5 (20)	1 (4)	0 (0)	0 (0)
High FA	20 (77)	6 (23)	0 (0)	0 (0)	0 (0)
Placebo	7 (25)	2 (7)	5 (18)	7 (25)	7 (25)

The larger number of deficient plasma and/or deficient RBC folate assays was used; plasma folate < 6.7 nmol/l and RBC folate < 315 nmol/l were considered deficient. Fisher's exact test, $p < 0.001$, was used to compare the 3 treatment groups with respect to the proportions of 0, 1, or more than 1 occurrence of deficient folate levels.

Table 2. Chronic hyperhomocysteinemia in MTX treated patients with RA receiving low and high dose folic acid supplements and placebo.

	Number (%) of Patients with 0, 1, 2, 3, or 4 Elevated Homocysteine Assays During Followup Visit				
	0	1	2	3	4
Group					
Low FA	20 (80)	4 (16)	1 (4)	0	0
High FA	25 (96)	0	0	0	1 (4)
Placebo	15 (53)	2 (7)	5 (18)	3 (11)	3 (11)

Homocysteine levels > 15 μmol/l are considered elevated. Fisher's exact test, $p < 0.001$, was used to compare the 3 treatment groups with respect to the proportions of patients who had 0, 1, or more than 1 occurrence of elevated plasma homocysteine levels.

mocysteinemia was found in less than 5% of the subjects in the folic acid supplemented groups ($p < 0.001$).

Figure 3 shows that homocysteine levels decreased with time for the folic acid supplemented groups ($p < 0.001$ low FA; $p = 0.023$ high FA), but not in the placebo group. The rate of decline was -4.64 µmol/l/year for the low FA, -2.88 µmol/l/year for the high FA group, and 0.20 µmol/l/year for the placebo group.

Dietary intake and correlations with biochemical indices. Mean dietary folate, vitamin B_{12}, and vitamin B_6 intakes were not significantly different within groups at any visit or between groups. Dietary folate, vitamin B_{12}, and vitamin B_6 intakes were not significantly correlated with homocysteine levels across groups or for any individual treatment group.

Correlations of vitamin levels with homocysteine. At Visit 1 (baseline), plasma folate ($N = 79$; $r = -0.334$, $p = 0.002$), RBC folate ($N = 79$; $r = -0.344$, $p = 0.003$) were significantly correlated with plasma homocysteine levels. At Visit 1 vitamin B_6 and vitamin B_{12} status was not correlated with baseline plasma homocysteine levels. In the placebo group, there was a relatively weak negative correlation between plasma folate and plasma homocysteine levels at Visits 2–5 ($r = -0.23$, $p = 0.025$).

DISCUSSION

MTX is increasingly being used for treatment of different chronic disorders, including RA; therefore, the metabolic consequences of chronic administration are important[1–6]. Low dose MTX therapy, given over a one year period, adversely affects both plasma and RBC folate levels. This effect was most pronounced in the placebo treated group and produced chronic deficient blood folate levels. We have shown that the C_1 index, a direct measure of the folate dependent formation of serine from formate and glycine in leukocytes, is lower in patients with RA treated with MTX compared to patients with RA not receiving MTX[39]. Folic acid supplementation (both 5 and 27.5 mg per week) prevents the decrement in folate status[8].

Our data indicate that both plasma and RBC folate are primary determinants of homocysteine levels in patients with RA taking MTX; this agrees with the observations in populations not treated with antifolates[9,10]. There were no significant relationships between vitamin B_6 and vitamin B_{12} levels and homocysteine levels at Visit 1. This indicates that folate was the predominant vitamin factor regulating the plasma homocysteine levels in our group of patients with RA, before the initiation of MTX therapy. The finding of no association between vitamin B_6 and homocysteine levels differs from Roubenoff, *et al*, who found low pyridoxal phosphate levels in patients with RA[40]. We did not have a control group for comparison in this trial and methionine loading tests were not performed during this protocol, which may explain differences. In addition, there were no significant relationships between dietary folate intakes and plasma homocysteine levels in any of the groups at Visits 2–5. This may reflect that naturally occurring food folate has been shown to be relatively ineffective at increasing folate status and perhaps altering homocysteine levels[41]. On the other hand, our previous findings suggest that food folate intakes of > 400 µg/day are effective in lowering the probability of MTX toxicity[8].

A substantial number of patients in the placebo group developed MTX induced chronic hyperhomocysteinemia, a condition largely prevented by folic acid supplementation in the other treatment arms. There is evidence suggesting that hyperhomocysteinemia is directly involved in the etiology of vascular atherosclerotic disease[17–20], early onset venous and arterial occlusion[21–23], coronary artery disease[24–26], and carotid artery stenosis[27]. It follows that MTX treated patients with RA should also have an increased risk for coronary artery disease, peripheral vascular disease, and cerebrovascular disease. There are data supporting the relationship between hyperhomocysteinemia and thrombosis in patients with systemic lupus erythematosus[42]. Hyperhomocysteinemia has also been observed in a small group of patients with RA in Sweden[43]. Specific epidemiological data for supporting the above assertion are, with one exception[44], lacking in MTX treated patients. In the one albeit small study specifically addressing MTX as a risk factor for cardiovascular disease, MTX treated patients with RA were compared to the healthy population[44]. Standardized mortality rates were 2.9 and 1.4, respectively, but the confidence intervals encompassed the unity (0.6–8.6 and 0.6–2.6, respectively) and by definition cannot be regarded as statistically significant, which likely relates to the relatively small size of the cohort studied.

Hyperhomocysteinemia in the low dose MTX treated population may be due to the interference in folate metabolism by the drug itself or to drug induced folate deficiency, or a combination of both[45]. It is intriguing that low serum folate levels, per se, were found to be associated with increased risk of fatal heart disease in the Nutrition Canada survey[26].

It is known that folic acid supplementation lowers homocysteine levels in an "at risk" population, such as in patients during the post-myocardial infarction period[46]. Based on our findings, low dose MTX treated patients should be added to the "at risk" population for increased cardiovascular risk due to hyperhomocysteinemia. The cereal grain fortification mandated by the Food and Drug Administration, in large part to prevent neural tube defects, also has the possibility of benefiting the population taking antifolate drugs by preventing toxicity, increasing folate levels, and decreasing homocysteine levels[7,8,47,48]. Folic acid supplements rather than food folates may have a more predictable effect in increasing blood folate levels[41]. It would still seem prudent to evaluate vitamin B_{12} status before prescribing longterm folic acid supplementation because of a high prevalence of

vitamin B_{12} deficiency in the RA population[49] and concerns of masking the nutritional anemia of B_{12} deficiency[47,50]. The prevalence of the thermolabile mutation in methylenetetrahydrofolate reductase or heterozygosity for cystathionine-β synthase deficiency in the population taking low dose MTX may merit further investigation, since these conditions produce hyperhomocysteinemia and may have an effect on recommended folic acid supplement doses[51–54].

We acknowledge that problems in sample handling can produce spurious increases in the homocysteine levels[55–57]. It has been shown that samples for homocysteine levels should be placed immediately on ice to prevent homocysteine from leaching from RBC into plasma and falsely elevating plasma homocysteine levels. In our study, samples were generally centrifuged and frozen at −70°C within 30 min so that artefactual hyperhomocysteinemia should be equal in all groups. All patient groups were enrolled simultaneously; therefore the blood was stored about the same length of time in all groups before analysis. It is also very unlikely that sample handling could account for the observation of chronic hyperhomocysteinemia found only in the placebo group.

These observations regarding homocysteine levels during low dose MTX therapy do not agree with our previous observations that homocysteine levels did not become elevated after 6 months of low dose MTX therapy[58]. The timing of blood sampling may have been a factor in the previous study; most samples were drawn 5–7 days after MTX administration, when homocysteine levels have been shown to return to normal after a low dose of MTX[45]. The failure to detect MTX induced hyperhomocysteinemia in our previous study may also be due to a shorter trial of MTX in the previous study (6 months vs one year) and a smaller number of patients in that study.

In summary, there are now 3 reasons to recommend low cost folic acid supplements in patients receiving longterm, low dose MTX therapy: (1) folic acid supplementation lessens MTX toxicity[7,8]; (2) folic acid supplementation should prevent chronic blood folate deficiency during therapy; and (3) folic acid supplementation lowers the prevalence of chronic hyperhomocysteinemia, which has been linked to cardiovascular disease risk in the general population.

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Role of Folic Acid in Modulating the Toxicity and Efficacy of the Multitargeted Antifolate, LY231514

JOHN F. WORZALLA, CHUAN SHIH and RICHARD M. SCHULTZ

Cancer Research Division, Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46285, U.S.A.

Abstract. *We studied the effects of folic acid on modulating the toxicity and antitumor efficacy of LY231514. Using several human tumor cell lines adapted to growth in low folate medium, folic acid was shown to be 100- to 1000-fold less active than folinic acid at protecting cells from LY231514-induced cytotoxicity. The lethality of LY231514 was compared in mice maintained on standard diet or low folate diet. The LD50 occurred at 60- and 250-fold lower doses of LY231514 in DBA/2 and CD1 nu/nu mice, respectively, maintained on low folate diet compared to standard diet. The L5178Y/TK-/HX- murine lymphoma was much more sensitive to the antitumor action of LY231514 compared to wild type L5178Y-S tumors. For mice on low folate diet, LY231514 at 0.3 and 1 mg/kg (qd x 10, i.p.) produced 100% inhibition of L5178Y/TK-/HX- lymphoma growth, and significant lethality occurred at ≥ 3 mg/kg. For mice on standard diet, LY231514 produced >95% inhibition of tumor growth at 30 to 300 mg/kg, but all mice died at 800 mg/kg. Folic acid supplementation was demonstrated to preserve the antitumor activity of LY231514 while reducing toxicity. The combination of folic acid with LY231514 may provide a mechanism for enhanced clinical antitumor selectivity.*

LY231514 is a structurally novel antifolate antimetabolite that possesses the unique 6-5-fused pyrrolo[2,3-d]pyrimidine nucleus (1) instead of the more common 6-6-fused pteridine or quinazoline ring structure. The primary mode of antitumor activity for LY231514 has previously been ascribed to inhibition of thymidylate synthase (TS) (1, 2). However, several lines of evidence suggest that multiple enzyme-inhibitory mechanisms are involved in cytotoxicity, hence the acronym MTA (multitargeted antifolate): 1) the reversal pattern for MTA in human leukemia and colon carcinoma cell lines demonstrates that although TS may be a major site of action for LY231514 at concentrations near the IC50, higher concentrations can lead to inhibition of dihydrofolate reductase (DHFR) and/or other enzymes along the purine de novo pathway (3); 2) MTA is an excellent substrate for folylpolyglutamate synthetase, and the K_i values of the pentaglutamate of LY231514 are 1.3, 7.2, and 65 nM for inhibition against TS, DHFR and glycinamide ribonucleotide formyltransferase (GARFT), respectively (3); 3) intracellular concentrations of LY231514 and its polyglutamates can exceed 40 μM in CCRF-CEM cells when ^{3}H-labeled LY231514 was used (R.M. Schultz, unpublished observation); and 4) early clinical studies demonstrated that patients who had previously failed to respond to ZD1694 and 5-fluorouracil/leucovorin treatment responded to LY231514 (4; DA Rinaldi, personal communication).

Several animal studies have indicated that folic acid supplementation in combination with antifolate cancer therapy can prevent delayed toxicity and enhance the therapeutic potential of the GARFT inhibitor lometrexol (5, 6) and the TS inhibitor 1843U89 (7). Unexpected delayed cumulative toxicity was observed in phase I studies with lometrexol, including thrombocytopenia, anemia, and mucositis (8). Additional clinical studies demonstrated the protective effects of folic acid against lometrexol toxicity in humans (9). Morgan and coworkers (10) concluded that a daily supplement of 1 mg of folic acid during low-dose methotrexate therapy in patients with rheumatoid arthritis was useful in lessening toxicity without altering efficacy. In the present communication, we investigated the effects of folic acid on the antitumor activity and lethality of LY231514 in mice.

Materials and Methods

Reagents. Folic acid, folinic acid (leucovorin), and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The disodium salt of LY231514 was synthesized at Eli Lilly and Co. (1).

Cell lines. Human CCRF-CEM leukemia cells were obtained from St. Jude Children's Research Hospital (Memphis, TN, USA). Human IGROV1 ovarian carcinoma cells were generously supplied by Dr.

Correspondence to: Richard M. Schultz, Cancer Research Division, DC 0546, Lilly Research Laboratories, Indianapolis, IN 46285, USA. Phone (317) 276-5508; fax (317) 277-3652; E-mail Schultz_Richard_M@Lilly.Com

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Barton Kamen (Univ. of Texas Southwestern Medical Center, Dallas, TX, USA). GC3 human colon carcinoma cells were obtained from Dr. Janet Houghton, St. Jude Children's Research Hospital. Human KB epidermoid carcinoma cells were purchased from the American Type Culture collection (ATCC, Rockville, MD, USA). The human LX-1 lung carcinoma cell line was established at Lilly from xenograft tissue. These cell lines were adapted to folic acid-free RPMI-1640 medium containing L-glutamine and 25 mM HEPES buffer (Whittaker Bioproducts, Walkersville, MD, USA) and supplemented with 10% dialyzed fetal calf serum (Hyclone Laboratories, Inc. (Logan, UT, USA) and 2 nM folinic acid. The L5178Y/TK-/HX- murine lymphoma cell line was obtained from Eli Lilly Department of Genetic Toxicology (Greenfield, IN, USA). The tumor is a double mutant, deficient in thymidine kinase and hypoxanthine phosphoribosyl transferase. It was cultured in RPMI-1640 medium supplemented with 10% horse serum. The L5178Y-S wild type lymphoma cell line was obtained from ATCC and routinely cultured in Fischer's medium (Whittaker Bioproducts) supplemented with 10% horse serum and 1 mM sodium pyruvate. All cell lines were tested and found free of mycoplasma contamination by the ATCC.

In vitro cytotoxicity testing. We used a modification of the original MTT colorimetric assay described by Mosmann (11) to measure cell cytotoxicity. The human tumor cells (previously adapted to growth in low folate (2 nM folinic acid) medium) were seeded at 1×10^4 cells in 80 µl of assay medium/well in 96-well flat-bottom tissue culture plates (Costar, Cambridge, MA, USA). Assay medium consisted of folic acid-free RPMI-1640 medium supplemented with 10% dialyzed fetal calf serum and 2 nM folinic acid. Well 1A was left blank (100 µl of growth medium without cells). Various levels of folic or folinic acid (0.1 to 100 µM) were added to the wells and incubated for 2 hours prior to addition of LY231514. LY231514 was prepared in Dulbecco's phosphate-buffered saline (PBS) at 1 mg/ml, and a series of two-fold dilutions were subsequently made in PBS. Aliquots (10 µl) of each concentration were added to triplicate wells. Plates were incubated for 72 hours at 37°C in a humidified atmosphere of 5% CO_2-in-air. MTT was dissolved in PBS at 5 mg/ml. Following incubation of plates, 10 µl of stock MTT solution was added to all wells of an assay, and the plates were incubated at 37°C for two additional hours. Following incubation, 100-µl dimethyl sulfoxide was added to each well. Following thorough formazan solubilization, the plates were read on a Dynatech MR600 reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Mice. Female CD 1 nu/nu mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Female DBA/2 mice were purchased from Taconic (Germantown, NY, USA). Mice weighed 20 to 25 grams at the beginning of the studies. Mice were housed in temperature and humidity controlled rooms. Mice were fed either standard laboratory rodent chow (Purina Chow #5001) or folic acid-deficient diet containing 1% succinylsulfathiazole (Purina Chow #5831C-2); both diets were purchased from Ralston Purina Co. (St. Louis, MO, USA). The average content of folates from natural sources in both diets was found to be 0.03 ppm, whereas the standard diet was analyzed to contain 7.3 ppm of added folic acid. It was estimated that mice on a standard diet ingested 1 to 2 mg/kg/day of folates, while mice on a low folate diet ingested 0.001 to 0.008 mg/kg/day. In some studies, mice received solubilized folic acid once a day by oral gavage. Food and water were provided ad libitum.

In vivo antitumor drug testing. L5178Y-S and L5178Y/TK-/HX were established and characterized in vivo for tumor growth in syngeneic DBA/2 mice. Cells derived from in vitro culture were washed twice by centrifugation (300 *g* for 10 minutes) in serum-free medium. Recipient DBA/2 mice were shaved and inoculated subcutaneously in the axillary region with 2×10^6 cells in 0.5 ml serum-free RPMI-1640 medium. LY231514 treatment was administered i.p. on a daily schedule for ten days and initiated on the day after tumor implant. LY231514 was dissolved in 0.9% sodium chloride solution. All animals were weighed at the beginning and end of drug treatment. Two-dimensional measurements (width and length) of all tumors were taken using digital electronic calipers interfaced to a microcomputer (12). Tumor weights were calculated from these measurements using the following formula:

$$\text{Tumor weight (mg)} = \text{tumor length (mm)} \times \text{tumor width (mm)}^2/_2$$

Percent inhibition of tumor growth was determined by comparing the tumor weight in treated groups to that of controls. No group was included in the analysis for therapeutic activity in which deaths attributable to drug toxicity exceeded 20% of the treated group.

Results

In vitro protective effect of folic or folinic acid for the cytotoxic activity of LY231514. We tested the ability of folic and folinic acid to protect human carcinoma and leukemia cells from LY231514-induced cytotoxicity. Previous studies demonstrated that the antiproliferative activity of LY231514 for CCRF-CEM leukaemia cells was completely reversed by the addition of leucovorin (0.05 to 16 µM) in a competitive manner (1). This suggested that LY231514 competed with natural reduced folate cofactors both at transport and intracellular folate levels and acted as a pure folate antagonist. In addition, we have reported that LY231514 is primarily transported via the reduced folate carrier (RFC) in human cell lines (3). For the current studies, we utilized tumor cell lines that had been adapted over >4 weekly passages to growth in low folate (2 nM folinic acid) media. Varying concentrations of folic and folinic acid were added to these adapted cells 2 hours prior to LY231514 exposure. As shown in Table I, the sensitivity to LY231514 cytotoxicity (IC_{50}) of low folate medium-adapted cells ranged from 3.6 nM (CCRF-CEM leukemia) to 44 nM (IGROV1 ovarian carcinoma). In addition, Table I shows the ability of folic acid and folinic acid to modulate the cytotoxic activity of LY231514 in five different human tumor cell lines. Folic acid was approximately 100- to 1000-fold less active than folinic acid at protecting cells from LY231514-induced cytotoxicity. Folic acid required concentrations of 10 µM or greater to exert significant protection.

Enhanced lethality of LY231514 to mice with dietary restriction of folic acid. Dietary folate deprivation has previously been shown to markedly enhance the toxicity of lometrexol (5). To assess the importance of dietary folate in modulating the toxicity of LY231514, LD_{50} values were determined in mice maintained on standard diet (normal rodent laboratory chow) or on a special low folate diet (LFD). LFD mice have been shown to be significantly folate deficient in plasma and several tissues including liver and implanted tumors (13). Mice maintained on LFD for two weeks before intraperitoneal adminstration of LY231514 daily for 10 days were extremely sensitive to the toxic effects of LY231514 with LD_{50} values of 1.6 and 10 mg/kg for CD1 nu/nu and DBA/2 mice, respectively (Figure 1). In contrast, the LD_{50} values for CD1 nu/nu and DBA/2 mice maintained on standard diet

Table I. *In vitro protective effects of folic or folinic acid on LY231514-induced cytotoxicity.*

		Relative (-fold) Change in IC_{50}						
		Folic acid conc. in media[c]			Folinic acid conc. in media			
Cell line[a]	IC50 (nM)[b]	1 μM	10 μM	100 μM	0.1 μM	1 μM	10 μM	100 μM
IGROV1	44	1	14	25	28	370	>970	>970
KB	34	2	3	17		6	78	>1270
OC3	12	1	3	9		105	47	640
LX-1	4	1	3	6		6	82	1460
CCRF-CEM	4	1	4	22	2	22	130	4600

[a]Cells were adapted to >4 weekly passages in low folate (2 nM folinic acid) medium.
[b]Cytotoxicity was determined by MTT assay with 72 h exposure to LY231514. Data represent mean of triplicate determinations.
[c]Folic or folinic acid was added two hours prior to LY231514 addition.

were approximately 250- and 60-fold greater, respectively than mice on LFD.

Role of folic acid in the antitumor activity of LY231514 against the L5178Y murine lymphoma. High circulating thymidine levels in mice decrease the efficacy and toxicity of TS inhibitors in mice (14, 15). Unless a tumor model which cannot salvage thymidine is utilized in mice, only limited antitumor effects for specific TS inhibitors have been observed. LY231514 treatment (i.p., qd x10) produced modest activity against the wild type L5178Y-S murine lymphoma (Table II). In contrast, similar treatment of a variant of this line, L5178Y/TK-/HX-, produced potent tumor suppression (100% tumor inhibition on the day following the last drug treatment at 30 and 100 mg/kg per day) with 11 of 14 mice tumor-free on day 100 after tumor implantation. This tumor is deficient in both thymidine kinase as well as hypoxanthine-guanine phosphoribosyl transferase and consequently, cannot salvage either thymidine or the purines hypoxanthine and guanine. The exquisite sensitivity of the L5178Y/TK-/HX- tumor model to LY231514 treatment allowed us to evaluate the effect of low folate diet on the therapeutic activity of this compound. For mice on LFD, LY231514 at 0.3 and 1.0 mg/kg/day (i.p. qd ×10) produced 100% inhibition of tumor growth for tumors measured one day after the completion of a single course of drug treatment (Figure 2). As noted in Figure 1, higher drug levels yielded unacceptable toxicity. For mice on LFD that received a folate supplement of 15 mg/kg/day via oral gavage, significant inhibition of tumor growth was noted over a broad dose range (10 - 1000 mg/kg/dose). Moreover, 100% inhibition of tumor growth was observed at 30 to 1000 mg/kg/dose without any lethality. This antitumor dose response (with folate supplementation) was virtually identical to that observed for mice receiving standard diet. However, the lethality was signicantly greater for the mice on standard diet (lethality at 400 and 800 mg/kg/day of 10% and 100%, respectively). Mice on standard diet received approximately one-tenth of the amount of daily folic acid as the mice on LFD with 15 mg/kg/day supplemental folic acid.

Table II. *LY231514 antitumor activity against L5178Y/S wild type and L5178Y/TK-/HX-lymphoma.*

	Tumor Dose[a] (mg/kg)	% Tumor Inh.[b]	# Tumor-free/total day 10[c]	day 100
L5178Y/S				
	10	0	0/10	-
	30	8	0/10	-
	100	68	0/10	-
L5178Y/TK-/HX-				
	10	90	0/7	0/7
	30	100	5/7	5/7
	100	100	7/7	5/7

[a]LY231514 was administered i.p. on a qd x 10 schedule.
[b]Tumors were measured on the day following the last drug treatment.
[c]Days represent the number of days since therapy was initiated.

Discussion

The poor predictive value of mouse models for antifolate toxicity may be partially due to the fact that standard laboratory mouse diets contain high levels of folic acid. Previous data demonstrated that serum and RBC folate levels of mice maintained on a diet formulated without added folic acid fall to levels considered normal in humans (5, 13). In this paper, we demonstrate that mice fed a low folate diet for a short period (2 weeks) became 60- to 250-fold more sensitive

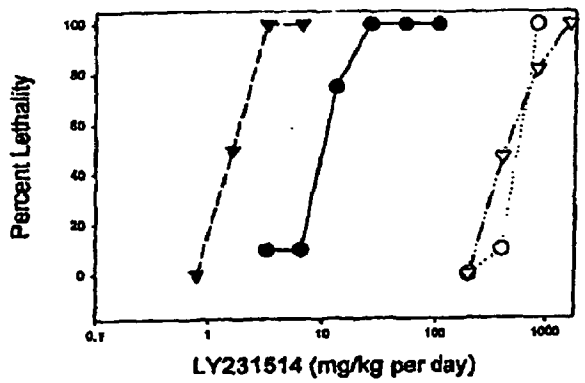

Figure 1. The toxicity of LY231514 in mice is increased by a folate-deficient diet. DBA/2 and CDI nu/nu mice were fed either a standard laboratory diet (○ and ▽, respectively) or a folate-deficient diet for 2 weeks prior to the first dose of LY231514 (● and ▼, respectively) and for the duration of the study. Groups of mice (> 10 animals/group) on each diet were given 10 daily doses of LY231514 i.p. at the indicated doses. The data present the percent lethality within 3 weeks after the last dose of LY231514.

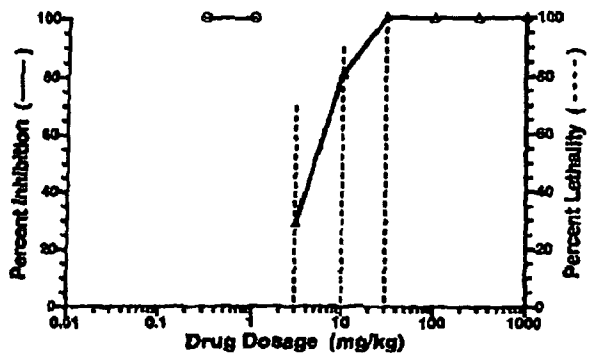

Figure 2. Antitumor activity of LY231514 therapy (i.p., qd ×10) against L5178YTK- /HX- lymphoma for mice on low folate diet with no folate supplementation (○) and for mice on low folate diet that received 15 mg/kg/day daily folate supplementation (△). Vertical dashed lines represent percent lethality in mice on low folate diet with no folate supplementation. No lethality was observed in mice that received folate supplementation.

to the lethality of LY231514 than observed in mice fed standard laboratory diet (Figure 1). The antifolate GARFT inhibitor, lometrexol has previously been shown to accumulate in the livers of folate-deficient mice, and this accumulation was diminished by the administration of folic acid to these animals (16). These investigators hypothesized that the substantial and unexpected toxicity of lometrexol in humans not given concurrent folic acid and in folate-deficient mice is due to the sequestration of drug in hepatic tissue, with the subsequent slow release of drug to the circulation at *toxicologically relevant concentrations*. The mechanism for this accumulation of lometrexol in liver probably involves metabolism to polyglutamate forms by the enzyme folylpoly-γ-glutamate synthetase (FPGS). In this regard, Mendelsohn and coworkers (6) demonstrated that liver produced the greatest response in elevated FPGS to low dietary folate of all tissues tested. A similar mechanism probably exists for the potentiation of LY231514 toxicity by folate-deficient diet, since this compound is an extremely efficient substrate for mouse liver FPGS (1). In addition, LY231514 requires polyglutamation for cytotoxic potency (3).

The uptake of natural reduced folate compounds and folate analogues into cells appears to involve membrane protein receptors of two different classes: a reduced folate/methotrexate carrier (RFC), which binds reduced folate in the micromolar range, and a high-affinity folate binding protein (mFBP), which preferentially binds to oxidized folate and other analogs with an affinity <1 nM (17). Studies using a panel of ZR-75-1 human breast sublines with differing transport properties have demonstrated a predominant role for the RFC in intracellular transport of LY231514 (3). Similarly, we now report that folic acid only weakly modulates the cytotoxic activity of LY231514 for various human leukemia and carcinoma cells adapted to low folate conditions (Table I). Some of these cells (KB and IGROV1) have previously been demonstrated to possess elevated levels of mFBP (18), further suggesting a minor role for mFBP in LY231514 transport.

LY231514 produced potent antitumor activity against the L5178Y/TK-/HX- lymphoma at 100-fold lower dose levels (0.3 and 1 mg/kg/day, Figure 2) in LFD mice relative to 30 and 100 mg/kg (Table II) in mice on standard diet. It is interesting to note that the LD_{50} was reduced 3000-fold for lometrexol in LFD animals, and antitumor activity could not be demonstrated even at low dose levels (5). In contrast, the shift in both LD_{50} and antitumor activity for mice on LFD compared to standard diet were of a similar magnitude (approximately 100-fold) for LY231514. However, LFD animals with high levels of folate supplementation demonstrated decreased lethality to LY231514 compared to conventional diet animals, suggesting that folate intake can be manipulated to achieve greater therapeutic effects. Oral folic acid dramatically decreased the toxicity of LY231514 and preserved antitumor activity (albeit at higher dose levels) in these mice (Figure 2).

Previous studies have demonstrated that the multitargeted antifolate, LY231514 has a unique biochemical and pharmacological profile. Exciting antitumor activity has been observed in phase I and II clinical trials, including responses in colon, breast, non-small cell lung and pancreatic cancers. More advanced and extensive clinical trials of LY231514 are currently in progress. The combination of folic acid with

LY231514 may provide a mechanism for enhanced clinical antitumor selectivity.

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9 8 7 6 5 4 3 2 1

TABLE 29-16. Toxicity Symptoms Reported to Be Associated with Chronic High Doses of Pyridoxine

Symptoms
Motor and sensory neuropathy; vesicular dermatosis on regions of the skin exposed to sunshine.
Peripheral neuropathy; loss of limb reflexes; impaired touch sensation in limbs; unsteady gait, impaired or absent tendon reflexes; sensation of tingling that proceeds down neck and legs
Dizziness; nausea; breast discomfort or tenderness
Photosensitivity on exposure to sun

Source: Leklem LE. Vitamin B6. In: Machlin LJ, ed. Handbook of vitamins. 2nd ed. New York: Marcel Dekker, 1991.

conditions of vitamin B_6 deficiency, the enzyme is not saturated by coenzyme *in vivo*, and the activity ratio will exceed 1.5 and 1.25, respectively.[223] An elevated erythrocyte AST (EAST) index or ratio is a commonly accepted indicator of inadequate B_6 nutriture.

An older procedure for determination of B_6 nutritional status is the tryptophan loading test. Urine is collected for 24 hours after ingestion of 2 to 5 g of L-tryptophan, and output of xanthurenic acid is measured. In vitamin B_6 deficiency, kynureninase activity is decreased, and kynurenine and 3-hydroxykynurenine accumulate. There is a resultant increase in excretion of tryptophan metabolites, including xanthurenic acid (see Fig. 29-20). A similar protocol is employed in the methionine loading test, with assessment of cystathionine excreted being used to evaluate B_6 status. Other widely used methods for vitamin assessment have included microbiologic and fluorometric assays.[225]

The concentration of plasma PLP is considered to be the best indicator of vitamin B_6 status, including tissue stores. The 4-pyridoxic acid content of a 24-hour urine reflects the production and excretion of the major metabolite of B_6. Reduced excretion of this urinary metabolite is one of the earliest indicators of a B_6 deficiency.

Direct assessment of B_6 levels is complicated by photosensitivity of the vitamers. HPLC methods for measurement of 4-pyridoxic acid levels in the urine or B_6 vitamers in the plasma are rapid, specific, and sufficiently sensitive to be clinically useful.[8,65,218] A sensitive and reliable procedure for determination of PLP by HPLC with electrochemical detection has been described.[47]

Also of interest is a radioenzymatic assay for direct measurement of PLP, based on activity of the PLP-dependent enzyme tyrosine decarboxylase from *Streptococcus fecalis*.[34] The commercially available apoenzyme is incubated with tritiated tyrosine and patient plasma. PLP in the specimen provides the required coenzyme, and the decarboxylated metabolite formed ([^{3}H]tyramine) is extracted and quantified by liquid scintillation counting.

Folates

Folates comprise a family of compounds derived from folic or pteroylglutamic acid.[192] All members of the family possess the double-ring structure pteridine (2-amino-4-hydroxy-6-methylpterin) joined by a methylene bridge to *para*-aminobenzoic acid (PABA). This parent compound is called *pteroic acid (Pte)*. PABA, in turn, is linked through a peptide bond to one molecule of glutamic acid, forming folic acid (FA) or pteroylglutamic acid (PteGlu; PGA) (Fig. 29-22). Conjugation with additional glutamic acid residues produces a series of polyglutamates. The bulk of the vitamin is present in the diet as folate polyglutamates. Enzymes requiring folic acid as a coenzyme catalyze chemical reactions involving the transfer and utilization of single carbon units. Nitrogen atoms at the 5 and 10 positions in the pteridine ring portion of the molecule are active in these single carbon unit transfers. The polyglutamate chain attaches the coenzyme to the apoenzyme. Double bond reduction and presence of various substituents serve to differentiate the various analogs of folic acid. Reduction of double bonds between ring positions 5–6 and 7–8 converts folic acid into tetrahydrofolic acid (THFA, or FH_4). The term *folate* is applied generically to the entire group of compounds. Use of the older generic descriptor, folacin, is no longer acceptable.

The most recently published folate RDAs are 180 μg for adult females and 200 μg for adult males.[181] For adolescents 150 μg is recommended. The minimal daily requirement for folate is approximately 50 μg for adults.[100] Use of oral contraceptive steroids can increase urinary excretion of folate. Increased vitamin intake may be required to offset the loss.[212,229] In pregnancy, the RDA is raised to 400 μg to maintain maternal folate reserves and adequately support normal fetal growth (see Table 29-4).[181] Megaloblastic anemia of pregnancy is commonly due to folate deficiency. Folic acid, even as much as 15 mg daily over several years, is reportedly not toxic in humans.[103] However, some data suggest that excessive intake of supplemental folate may interfere with intestinal absorption of zinc.[31,170]

The name *folate*, like the word foliage, is derived from the Latin word for leaf. Cruciferous vegetables, such as spinach, turnip greens, asparagus, broccoli, and brussels sprouts, are

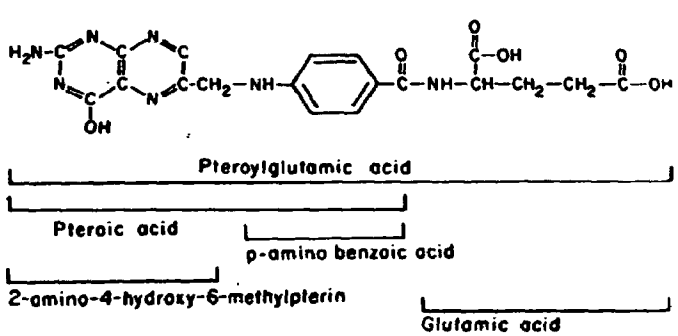


Figure 29-22. Folacin.

rich in folate. Folate is abundant in liver, kidney, whole-grain cereals, yeast, and mushrooms.[82] The vitamin is also synthesized by intestinal microflora. Prolonged cooking, particularly steaming and boiling, destroys most folate in foods. Infants receiving boiled formulas prepared with pasteurized, sterilized, or powdered cow's milk require folate supplementation.

Following ingestion, polyglutamates are enzymatically hydrolyzed to monoglutamates by action of conjugases in the mucosa of the small intestine (Fig. 29-23). Folate monoglutamates are rapidly absorbed and transported in the circulation mainly as the tetrahydrofolate (FH_4) derivative. The major form of folate in serum and red cells is 5-methyl-tetrahydrofolate (N^5-methyl-FH_4). Dihydrofolate reductase

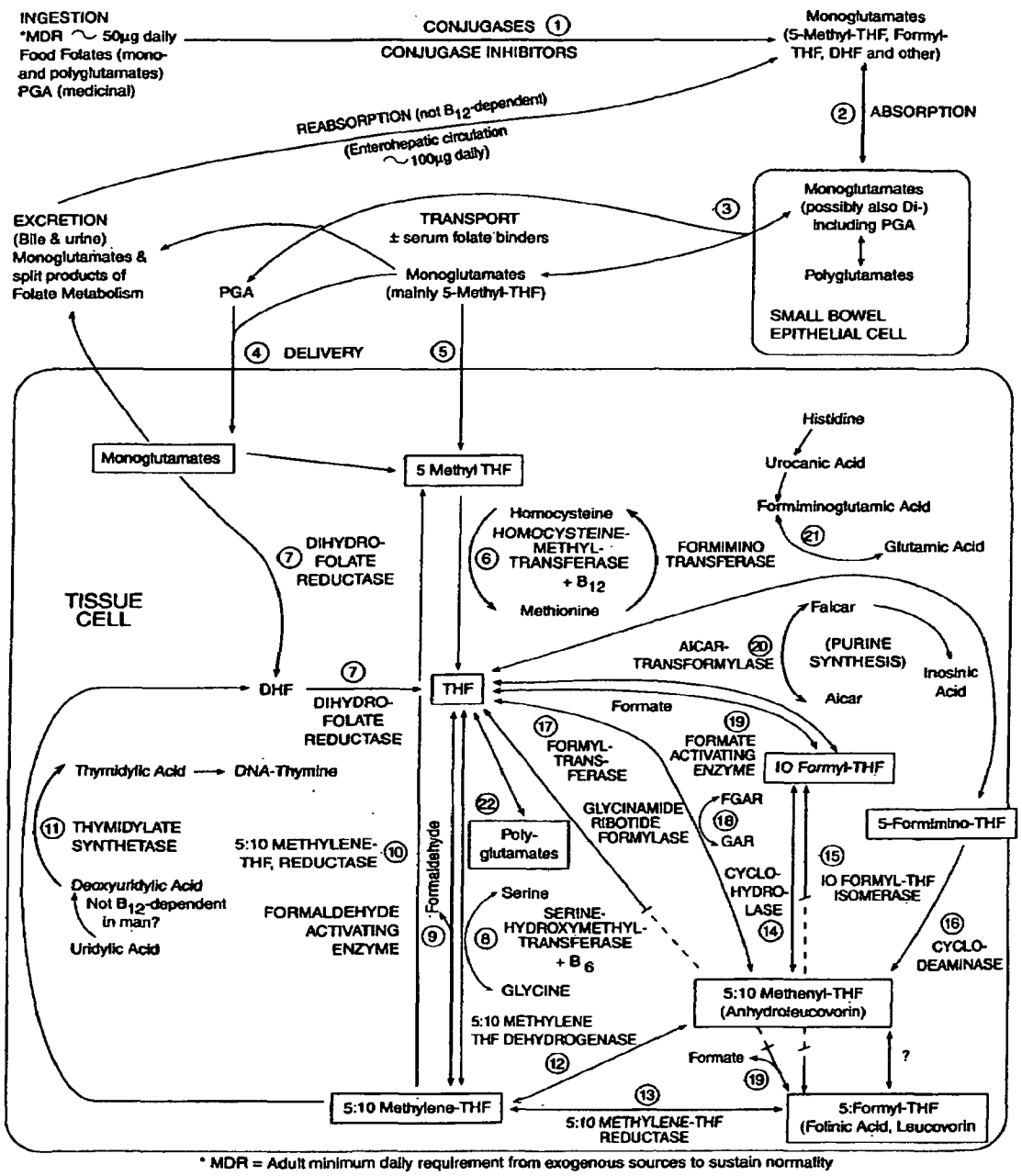


Figure 29-23. Flow chart of folate metabolism in humans. Circled numbers indicate individual steps in folate metabolism. *Source:* Herbert V, Das KC. Folic acid and vitamin B12. In: Shils ME, Olson JA, Shike M, eds. Nutrition in health and disease. 8th ed. Vol.1. Philadelphia, PA: Lea & Febiger, 1994.

catalyzes the enzymatic reduction reaction.[42] Folate may circulate in the free form or attached to low-affinity or high-affinity binders. Approximately two-thirds of folate is loosely bound to plasma proteins, including albumin, α_2-macroglobulin, and perhaps, transferrin. High-affinity folate-binding proteins have been purified from serum, milk, and cerebrospinal fluid. The role these specific proteins play in overall folate nutriture is not clear. The milk protein could facilitate intestinal uptake of folate. Presence of a folate-binding protein in the choroid plexus may account for the high CSF/serum ratio of the vitamin. Serum folate levels range from 3 to 25 ng/mL.[42] Marginal deficiency is suggested by concentrations from 3 to 5 ng/mL; levels above 5 ng/mL are interpreted as indicating adequate folate.[28,100] Recent efforts to establish a pediatric reference range show folic acid concentrations to be higher in children, especially in those less than 1 year of age, than in adults. During adolescence, a significant decrease in serum folic acid concentration has been noted.[102] Folate concentration in CSF ranges from 15 to 35 ng/mL.[42] While folate monoglutamates are the circulating and transport forms, polyglutamates are the primary intracellular storage forms of the vitamin.[100] Hepatic stores are believed to account for approximately 50% of the body's reserve of folate, predominantly as pentaglutamates. Other tissues with high concentrations of folate are the kidney and blood cells. RBC folate is almost entirely in the form of methylfolate pentaglutamates. Negative folate balance is indicated by erythrocyte vitamin levels less than 200 ng/mL; tissue depletion occurs when folate levels fall below 160 ng/mL.[100] Tissue folate-binding proteins are reported in granulocytes as well as the brush border of intestinal mucosa. Leukocyte folate ranges from 60 to 123 μg/L of WBCs.[42] Folate-requiring enzymes serve as intracellular folate binders. Excretion occurs in the bile and urine (Fig. 29-23).

Vitamin deficiency may be dietary in origin, associated with malabsorption, or drug-induced (Table 29-17). Nutritional folate deficiency is seen in infants raised on goat's milk, which has only about 10% of the concentration of the vitamin found in human or cow's milk.[181] Inborn errors of folate metabolism (*e.g.*, dihydrofolate reductase deficiency and congenital folate malabsorption) give rise to folate deficiency. Total parenteral nutrition using amino acid solutions, unsupplemented by folate, has been reported to induce acute depression of serum folate, marked by pancytopenia and megaloblastic anemia.[73] Folate malabsorption may occur in conditions such as Crohn's disease or ulcerative colitis. Ironically, sulfasalazine, which is used in the treatment of inflammatory bowel disease, impairs folate absorption. Vitamin deficiency may arise during anticonvulsant therapy with phenytoin or phenobarbital.[31] Other drugs that affect folate status include cycloserine, metformin, and cholestyramine. Antifolate medications are used in the treatment of a wide range of malignant and nonmalignant disorders.[100] Folate antagonists appear to bind irreversibly to the enzyme dihyrofolate reductase. Examples of such drugs are triamterene, a diuretic; pyrimethamine, an antimalarial; trimethoprim, an antimalarial as well as a potentiator of sulfonamides in the

TABLE 29-17. Diseases Treated with Drugs Known to Interfere with Folate Metabolism

Disease	Drug
Cancer, leukemia	Methotrexate
Psoriasis	Methotrexate
Rheumatoid arthritis	Methotrexate
Bronchial asthma	Methotrexate
Bacterial infection	Trimethoprim
Malaria	Pyrimethamine
Hypertension	Triamterene
Crohn's disease	Sulfasalazine
Gout	Colchicine
Epilepsy	Phenytoin
AIDS	Trimetrexate

Source: From Butterworth CE, Tamura T. Folic acid safety and toxicity: A brief review. Am J Clin Nutr 1989;50:353.

treatment of bacterial infections; and pentamidine, used in treatment of trypanosomiasis and leishmaniasis.[212] Pentamidine is also employed in the treatment of pneumonia, presumably due to protozoal infection. Pulmonary disease caused by *Pneumocystis carinii* occurs in 65% to 85% of all AIDS patients. The most common manifestation of this infection is pneumonia. Among the adverse reactions arising from standard pentamidine therapy in the treatment of this pneumonia is the development of folate deficiency. The cancer chemotherapeutic agent methotrexate (MTX) is an especially potent folate antagonist.[212] MTX may also be utilized in the treatment of psoriasis and rheumatoid arthritis. The acute toxicity of folate antagonists is due to their impairment of DNA synthesis. A pharmacologic amount (>0.4 mg/d) of folic acid may be administered as a "rescue dose" to patients receiving cancer chemotherapy.

In the U.S., inadequate folate nutriture is particularly common among those in lower socioeconomic groups.[28] Folic acid deficiency has been reported as the most common nutritional deficiency among low-income and institutionalized elderly.[28,167,216] Exposure to ethanol may alter the activity of intestinal brush border folate hydrolase (conjugase), causing malabsorption of the vitamin. Alcohol also interferes with hepatic processing of folate. Chronic alcoholism is a major cause of folate deficiency in the United States.

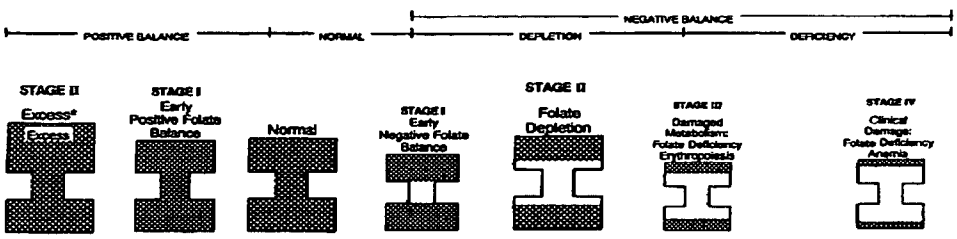
Tetrahydrofolate (THF) derivatives serve as cofactors for enzymes catalyzing the transfer of 1-carbon groups in methylation reactions necessary for a variety of biochemical reactions. The coenzyme forms of the vitamin include the following tetrahydrofolates: N^5-formyl-FH_4; N^{10}-formyl-FH_4; N^5-formimino-FH_4; N^5, N^{10}-methenyl-FH_4; N^5, N^{10}-methylene-FH_4; and N^5-methyl-FH_4.[192] The carbon units transferred by the coenzymes are present in varying states of reduction. Coenzyme activity appears to be greater with polyglutamate, rather than monoglutamate, forms of folate. Metabolic reactions requiring THF coenzymes include interconversion of serine and glycine; methionine synthesis from homocysteine (also a B_{12}-dependent pathway); histidine degradation to glutamic acid by means of formiminoglutamic

acid (FIGLU); purine biosynthesis; synthesis of the pyrimidine thymidylate, required in DNA synthesis; and the methylation of biogenic amines, including dopamine, tryptamine, serotonin, adrenaline, noradrenaline, and the generation/activation of formate.[42,192] A number of studies have suggested a role for folate in the reversal of preneoplastic conditions of cervical and lung cancers.[255]

Both biochemical and hematologic changes (Table 29-18) are characteristic of poor folate nutriture. The principal clinical feature of folate deficiency is megaloblastic anemia, but folate depletion may precede anemia by months. Other signs and symptoms of deficiency include anorexia, glossitis, nausea, diarrhea, hepatosplenomegaly, and hyperpigmentation of the skin.[42,64] Neurologic disorders also have been attributed to folate deficiency, although this is not routinely part of the clinical picture. Serum folate levels fall below normal after as few as three weeks of folate deprivation.[28] Deficiency of folate leads to inadequate synthesis of DNA and abnormal cell division. Morphologic evidence of the biochemical inadequacy includes bone marrow megaloblastosis, appearance of hypersegmented neutrophils in the peripheral blood, and macrocytosis of reticulocytes and platelets.[95] When red cell folate levels are less than 100 ng/mL (226.6 nmol/L), morphologic abnormalities in mature circulating red blood cells are detected with development of a macrocytic, normoblastic, or megaloblastic anemia. An elevated mean red cell volume and low hemoglobin are consequences of long-standing folate deficiency.[100] Elevation of certain metabolites in the serum serves as an early indicator of suboptimal levels of folate. For example, poor folate status can result in higher plasma levels of the atherogenic amino acid homocysteine.[49,123,180,267] Because of a lack of 5-methyltetrahydrofolate in amounts sufficient for the remethylation of homocysteine to methionine, homocysteine accumulates in the plasma. Toxic effects arising from excess homocysteine may be due to its interference with normal cross linking of collagen molecules, thereby disrupting or damaging the intimal surface of arteries. Low normal serum folate concentrations could therefore place an individual at increased risk of cardiovascular disease. Supplementation with modest doses of folate (1 to 5 mg/d) can often normalize elevated homocysteine concentrations.[25,124,216]

Approximately 6000 infants are born each year in the U.S. with neural tube defects. Maternal folic acid supplementation in early pregnancy reduces the risk of giving birth to an infant with a neural tube defect (*e.g.*, spina bifida or anencephaly) by as much as 75%. Because closure of the embryonic neural tube normally occurs by the sixth week of pregnancy, there is no deterrent advantage reported for women who begin supplementation after that point in time.[160,203,280] Folic acid fortification of basic foods, such as wheat flour, has been advocated. While this action would address the issue of women of child-bearing age receiving the vitamin in amounts sufficient to reduce the risk of fetal neural tube defects, it could create a medical dilemma for the elderly. It is estimated that pernicious anemia (PA) caused by malabsorption of vitamin B_{12} effects approxi-

TABLE 29-18. Sequence of Events in Developing Folate Deficiency. Earliest Abnormalities in Each Stage are Boxed



Serum Folate (ng/ml)	>10	>10	>5	<3	<3	<3	<3
RBC Folate (ng/ml)	>400	>300	>200	>200	<160	<120	<100
Diagnostic dU Suppression	Normal	Normal	Normal	Normal	Normal	Abnormal*	Abnormal*
Lobe Average	<3.5	<3.5	<3.5	<3.5	<3.5	>3.5	>3.5
Liver Folate (μg/g)	>5	>400	>3	>3	<1.6	<1.2	<1
Erythrocytes	Normal	Normal	Normal	Normal	Normal	Normal	Macroovalocytic
MCV	Normal	Normal	Normal	Normal	Normal	Normal	Elevated
Hemoglobin (g/dL)	>12	>12	>12	>12	>12	>12	>12
Plasma Clearance of Intravenous Folate	Normal	Normal	Normal	Normal	Normal	Increased	Increased

**Dietary excess of folate reduces zinc absorption.*

Due to hormonal effects (on receptors?), there may be folate deficiency (i.e. Stage III-IV negative balance) in cervical epithelial cells (a reversible lesion) (possibly precancerous?) when there is only early negative balance (i.e. Stage I-II negative balance) in the erythron (Ran et al. Blood, November 1990).

Source: Herbert B, Das K. Folic acid and vitamin B_{12}. In: Shils ME, Olson JA, Shike M, eds. Modern nutrition in health and disease. 8th ed. Philadelphia: Lea & Febiger, 1994.

mately 1 million Americans. Most of these individuals are older adults. A deficiency of either vitamin B_{12} or folic acid will create the same hematologic picture (*i.e.*, macrocytic, megaloblastic anemia). However, only a B_{12} deficiency will produce irreversible neurologic lesions. Folic acid supplementation can mask or delay diagnosis of B_{12} deficiency by restoring a normal hematologic picture without preventing the B_{12}-induced neurological disease. There is serious concern among health practitioners that widespread fortification of foods with folic acid would significantly increase the incidence of delayed diagnosis of vitamin B_{12} deficiency.[286]

Microbiologic assays of folates in serum, erythrocytes, and urine have been conducted in the clinical laboratory for many years.[110,171] Although not as rapid or convenient as newer radioassay procedures, microbiologic assay remains the reference method. The organism of choice is *Lactobacillus casei* (ATCC 7469), which utilizes all monoglutamate forms of folate, including the reduced form, 5-methyltetrahydrofolate, for growth. RBC folate is present as polyglutamates and must be converted to monoglutamates for analysis. Interference by antibiotics in the patient specimen presents a serious problem.

Indirect measurement of folate status has been attempted by employing a histidine loading test. Histidine is metabolized to glutamic acid by way of the intermediate formiminoglutamic acid (FIGLU). The final enzyme of this pathway, formiminotransferase, is folate-dependent. If folate is deficient, FIGLU accumulates. When an oral 2- to 15-g dose of histidine is administered to a folate-depleted patient, the amount of FIGLU excreted in the urine in the 8-hour period following the load is at least 5 to 10 times greater than the amount excreted by a folate-replete individual under the same conditions.[229]

Another approach in evaluating the adequacy of tissue folate to support normal biochemical function is by means of the deoxyuridine (dU) suppression test, which reflects slowed *de novo* DNA synthesis. The final step in the conversion of deoxyuridylate to thymidylate for DNA synthesis is folate-dependent. This test is generally abnormal in megaloblastic anemia due to both folate and B_{12} deficiency.[36]

There is concern over falsely low serum values for folate arising from oxidative destruction of the vitamin prior to analysis. To avoid vitamin loss, serum may be stored frozen or a reducing substance such as ascorbic acid may be added to the specimen. A recent study on the effect of light on serum folate concluded that specimens to be tested can be stored at room temperature for up to 8 hours in either a gel separator collection tube or in a polypropylene storage tube without substantial loss (<7%) of the vitamin. Folate specimens exposed to light for more than 8 hours should be redrawn.[154] Since folate levels of erythrocytes exceed serum levels by approximately 40-fold, it is essential that hemolyzed samples not be accepted for assay of serum folate. To measure erythrocyte folate levels, a hemolysate, prepared with an aqueous 1% ascorbic acid solution, is tested.[28]

Folate assessment by competitive protein-binding radioassay techniques is common. Tracers used are [^{125}I]folate or ^{3}H-PGA. The weak binding of folate to plasma proteins necessitates pretreatment or a denaturation step to liberate the vitamin before application of CPB techniques. Denaturation may be by heat (boiling) or by pH inactivation (no-boil). Incomplete denaturation of interfering proteins is sometimes experienced with a no-boil protocol. Radioligand assay procedures have been adapted for automated systems to permit simultaneous assays of serum folate and B_{12} after manual heat denaturation of endogenous protein binders.[45] Because these two vitamins are so closely linked in terms of biochemistry and metabolic function, it is important that they be evaluated together.[116]

HPLC is particularly useful in separating the various folate compounds. A competitive enzyme-linked ligand sorbent assay (ELLSA) for quantitation of folates has been described that offers promise for application in the clinical laboratory.[89]

Individuals with a folate deficiency will have a reduced capacity to convert homocysteine to methionine. Measurement of serum levels of homocysteine by modified techniques using capillary-gas chromatography and mass spectrometry have proven useful as a means of identifying suboptimal folate nutriture.[230] Totally automated methods, including a C_{18}-based HPLC assay and an FPIA requiring no pretreatment or chromatographic step, have been reported in the literature recently.[237a]

Vitamin B_{12}

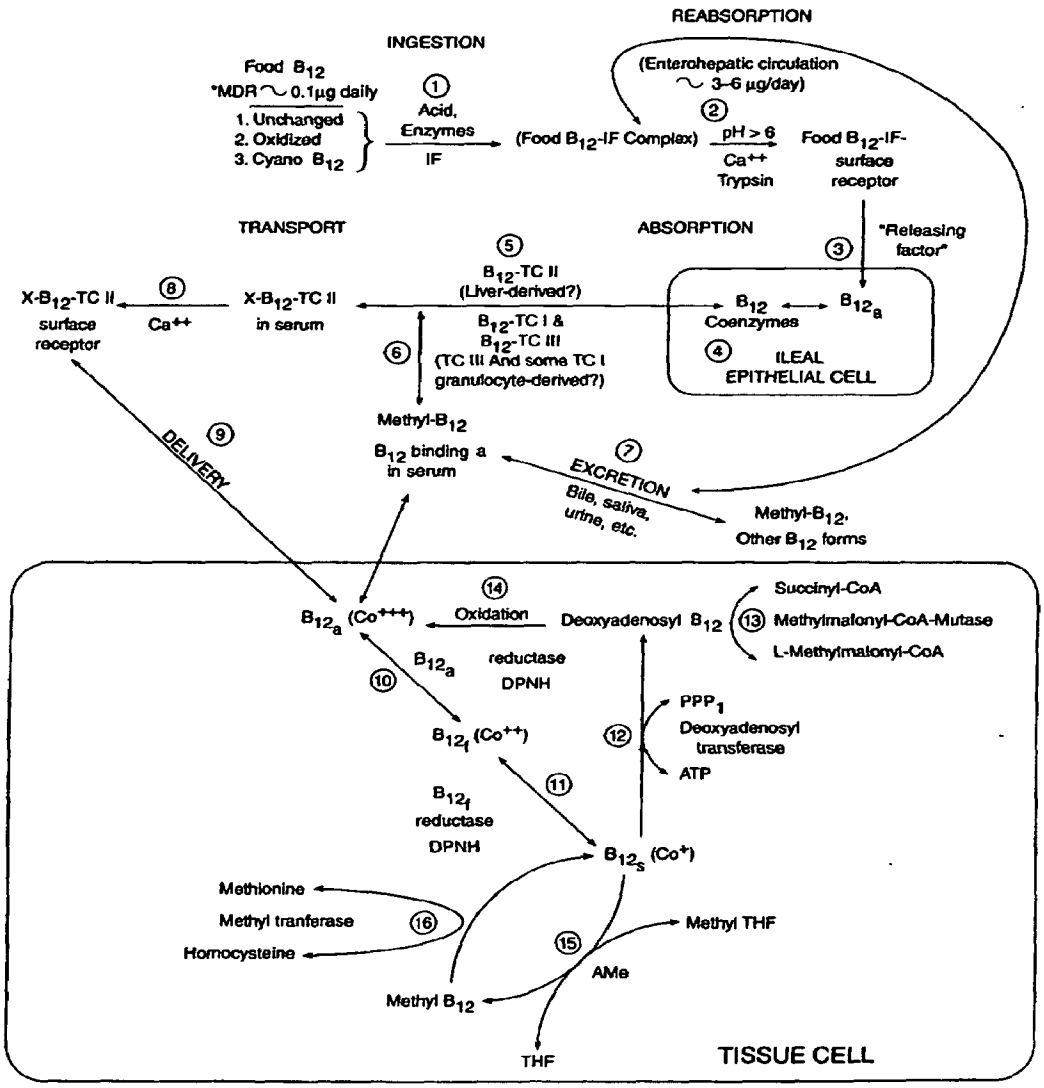
In 1948, vitamin B_{12} was isolated and crystallized for the first time by both American and British researchers.[206] IUPAC recommendations call for generic use of the name *cobalamin* for those vitamins that possess a cobalt-containing corrin ring attached to the nucleotide 5,6-dimethylbenzimidazole (see Table 29-11). Dimethylbenzimidazole is similar in structure to riboflavin. The corrin nucleus contains four substituted pyrrole rings and resembles the porphyrin nucleus of heme. Various ligands may be covalently linked to the cobalt atom, including cyanide anion (cyanocobalamin), hydroxyl group (hydroxocobalamin), methyl group (methylcobalamin), or 5′ deoxyadenosyl group (adenosylcobalamin).[192] The coenzyme forms of B_{12}, adenosylcobalamin and methylcobalamin, function as transmethylating agents.[42] Methylcobalamin accounts for approximately 75% of plasma vitamin B_{12}, whereas a similar percentage of liver B_{12} is in the form of adenosylcobalamin. B_{12} in erythrocytes and the kidney is also largely present as adenosylcobalamin. Smaller amounts of hydroxocobalamin and cyanocobalamin exist in body fluids and tissues.

The RDA for vitamin B_{12} is 2 μg for adults and adolescents of both sexes. In pregnancy and lactation, the requirement is increased, respectively, to 2.2 μg and 2.6 μg daily.[185] Although vegetable matter is devoid of vitamin B_{12}, it is present in animal products such as meat and dairy foods, including liver, eggs, milk, and cheese.[82] Microorganisms alone synthesize the vitamin, and animals, including humans, ultimately depend on this activity to furnish preformed B_{12}. Enteric microorganisms, mainly actinomycetes, synthesize B_{12} in the human colon, but it is not absorbed

through the mucosa in this region of the gastrointestinal tract.[29] B_{12} deficiency is rarely caused by poor nutrition. However, strict vegetarians, unless they receive B_{12} as a contaminant in food or supplement the diet, will develop a clinical deficiency.[96] The liver stores 50% to 90% of the body's B_{12}.[99] Reserves are relatively large, and it may require literally years for the classic features of deficiency to appear, even in complete absence of vitamin intake.

Vitamin B_{12} is absorbed in the intestine, depending primarily on the availability of intrinsic factor (IF), a glycoprotein secreted by gastric parietal cells (Fig. 29-24). These same cells secrete hydrochloric acid. Impaired absorption due to lack of intrinsic factor in gastric secretions gives rise to the clinical condition known as *pernicious anemia (PA)*. Achlorhydria, which diminishes B_{12} absorption, and PA, associated with atrophy of the gastric mucosa, are most common among individuals over 60 years of age. An extremely small percentage of vitamin B_{12}, probably less than 1%, is absorbed passively throughout the intestine, independent of IF complex formation. A diffusion-type mechanism for vitamin uptake, not mediated by IF, also seems to operate when large amounts (100–300 μg) of B_{12} are supplied.

The four common forms of cobalamin bind equally well to IF.[41] In the ileum, IF-B_{12} complex binds to specific membrane receptors of the mucosal brush border. A pH above 6 and the presence of calcium ions are required to promote vitamin absorption. Upon transiting the mucosal cell, vitamin B_{12} is released into the portal circulation. Plasma B_{12} is



* MDR = Adult minimum daily requirement from exogenous sources to sustain normality

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Figure 29-24. Flow chart of cobalamin (B_{12}) metabolism. Circled numbers identify individual metabolic steps. *Source:* Herbert B, Das K. Folic acid and vitamin B_{12}. In: Shils ME, Olson JA, Shike M, eds. Modern nutrition in health and disease. 8th ed. Philadelphia: Lea & Febiger, 1994.

bound by members of a group of carrier globulins, the transcobalamins (TC). Transcobalamin II (TCII) serves as primary transport protein for distribution of newly absorbed vitamin B_{12} to the tissues.[99] All cells that synthesize DNA possess surface receptors for TCII. One of the earliest detectable signs of a negative B_{12} balance is reportedly a decrease in serum holotranscobalamin (TCII + cobalamin).[96] Vitamin B_{12} also binds to haptocorrin, a circulating storage protein. The only receptors for haptocorrin are on B_{12} storage cells (*e.g.*, liver and reticuloendothelial cells). Other protein binders of B_{12} have been identified in body fluids, including serum, saliva, tears, milk, colostrum, cerebrospinal fluid, and gastric juice, as well as in blood cells. These endogenous proteins (TCI and TCIII) have been collectively designated as *R proteins* because of their rapid migration during electrophoresis. R proteins bind both biologically active cobalamin and inactive analogs. The physiologic function of these binding proteins is not clear, but they do not facilitate ileal absorption of the vitamin.[231] The therapeutic form of vitamin B_{12} is cyanocobalamin. If it is administered subcutaneously or intramuscularly, the need for IF-mediated intestinal absorption is bypassed. While the treatment of PA with oral B_{12} megadose therapy is more common in Europe than in the U.S., it has proven to be successful.[133] When given orally, in excess, enough B_{12} is absorbed even in the absence of IF to meet the requirements of most patients. Even in megadoses, cobalamin is reported to be nontoxic.

In humans, two enzymes are known to be vitamin B_{12}–dependent: 5-methyltetrahydrofolate (5-methyl-THF) homocysteine methyltransferase and methylmalonylcoenzyme A mutase.[42] Methylcobalamin functions as coenzyme for a methyltransferase reaction in methionine synthesis. The coenzyme form of folate, 5-methyl-THF, donates a methyl group to cobalamin, which transfers it to homocysteine, forming a new amino acid, methionine. Methionine is subsequently metabolized to succinyl-CoA. Thus, both folate and B_{12} participate in methionine synthesis. In the process, tetrahydrofolate (THF), required for synthesis of thymidylate in DNA, is regenerated from 5-methyl-THF. Vitamin B_{12} acts as a methyl receiver to prevent "trapping" of folate as the methylated tetrahydrofolate.[192] Interference with nucleotide synthesis impairs erythropoiesis and leads to development of megaloblastic anemia due either to deficiency of B_{12} or folate. The interrelationship of folate and B_{12} is also seen in a cobalamin requirement for folate uptake by cells.[82] In this instance, a folate deficiency may occur indirectly because of inadequate levels of B_{12}.

Adenosylcobalamin is required by the enzyme methylmalonyl-coenzyme A mutase for rearrangement of 1-methylmalonyl-CoA to succinyl-CoA. Succinyl-CoA is further metabolized through the tricarboxylic acid cycle. In states of B_{12}, but not folate, deficiency, methylmalonyl-CoA is not converted to succinyl-CoA, and methylmalonic acid (MMA) excretion in the urine is increased. In summary, B_{12} functions in oxidative degradation of amino acids and, since methionine is a glycogenic amino acid, in carbohydrate metabolism.[192] Fatty acids with odd numbers of carbon atoms are oxidized by a pathway requiring methylmalonyl-CoA mutase activity. Thus, B_{12} is also essential for normal lipid metabolism.[192] Inadequate supplies of cobalamin will disrupt lipid synthesis. This, along with decreased availability of adenosyl methionine needed for myelin protein formation, could explain the neurologic complications, including demyelination and degeneration of the central nervous system and the optic and peripheral nerves, seen in B_{12} deficiency.

Deficiency of B_{12} may be due to dietary absence, as among strict vegetarians, increased requirements, as in pregnancy, malabsorption due to disease, drug-induced interference (Table 29-19), or intrinsic factor and transport protein inadequacies.[99] Low cobalamin plasma levels are reported in patients with sprue, Crohn's disease, regional enteritis, pernicious anemia, gastric or intestinal resection, multiple myeloma, IF-blocking antibodies, or serum gastric parietal cell autoantibodies. Gastric and intestinal bacterial overgrowth may contribute to cobalamin malabsorption. Up to 25% of the geriatric population may be afflicted with chronic atrophic gastritis. Occurrence of this condition increases with age and may account for the widely reported low serum cobalamin concentrations among the elderly.[2,143,198,263] Studies have shown low serum cobalamin in 10% to 50% of elderly, depending upon variables such as the specific population of older adults examined, assay techniques employed, and cut-off values used to define risk of deficiency. The prevalence of cobalamin deficiency was found to be at least 12% in a large sample of ambulatory older adults when deficiency was defined by a serum cobalamin concentration <258 pmol/L and elevation of one or both of the metabolites MMA and homocysteine. Many elderly with "normal" serum vitamin levels were metabolically deficient in B_{12} or folate.[142] Impaired intestinal absorption of B_{12} has been reported in patients taking anticonvulsants, neomycin, *para*-aminosalicylic acid, phenformin, and cholestyramine, and also has been reported in alcoholics.[27] Controversy continues over reports that megadoses of ascorbic acid may lead to inactivation of vitamin B_{12} and destruction of IF.[27,96]

Clinical features of B_{12} deficiency generally include both hematologic (*e.g.*, macrocytic anemia, megaloblastosis, hypersegmentation of neutrophils) and neurologic (*e.g.*,

TABLE 29-19. Cobalamin-Drug Interactions

Drug	Mechanism of Interaction
Aminosalicylic acid (PAS)	Decreased absorption
Colchicine	Malabsorption
Neomycin	Malabsorption
Guanidines	Decreased absorption
Metformin	Decreased absorption
Phenformin	Decreased absorption
Potassium chloride	Decreased absorption
Nitrous oxide	Interferes with B_{12} metabolism
Fiber	Enhances excretion

Source: Ellenbogen L, Cooper BA. Vitamin B_{12}. In: Machlin LJ, ed. Handbook of Vitamins. 2nd ed. New York: Marcel Dekker, 1991.

peripheral nerve degeneration) manifestations. The hematologic picture is identical in both B_{12} and folate deficiency due to abnormal replication of DNA in hematopoietic tissue. Especially among the elderly, neuropsychiatric disorders may be the primary or only indication of cobalamin deficiency.[141] Numbness, tingling, and weakness of extremities are frequent early neurologic symptoms of vitamin B_{12} deficiency. Vision may be impaired. Spinal cord degeneration leads to changes in tendon reflexes and difficulty in walking. Cognitive dysfunctions include poor memory, loss of mental alertness and confusion, marked personality and mood changes, and, in rare instances, delusions and hallucinations may develop. Research is in progress to determine what, if any, relationship exists among serum cobalamin levels, normal aging, and the occurrence of dementia or Alzheimer's disease.[10,51] Some cognitive and hematopoietic dysfunctions found in AIDS patients have been reversed by vitamin B_{12} therapy. Elevated serum homocysteine concentrations due to vitamin deficiency may play a part since, in excess, the amino acid is both neurotoxic and vasculotoxic.[96]

Limited observations suggest that osteoblast activity depends on cobalamin and that bone metabolism is affected by cobalamin deficiency. Cobalamin-deficient patients were reported to have lower alkaline phosphatase and osteocalcin levels than controls. Osteocalcin, a vitamin K–dependent bone-specific protein, is synthesized by osteoblasts. Its concentration in plasma reflects the rate of bone formation. If so, not only bone marrow cells but also adjoining skeletal cells could be affected in B_{12} deficiency.[37] The osteopenia of aging may be related to an inadequate supply of vitamin B_{12}.[37]

Pernicious anemia (PA), a common cause of vitamin B_{12} deficiency, primarily affects the elderly. Diagnosis of PA by assessment of B_{12} intestinal absorption may be accomplished by measuring urinary excretion of ^{57}Co-labeled vitamin in the Schilling test.[42] An oral dose of ^{57}Co-B_{12} is administered along with a parenteral injection of nonlabeled B_{12}. Labeled B_{12} absorbed in the intestine enters the pool of unlabeled vitamin in the plasma, and both forms are excreted in the urine. The percentage of the oral dose appearing in the urine in 24 hours is calculated. Normal B_{12} absorption is indicated when more than 10% of the oral dose is excreted by the patient. Reduced excretion of radioactive B_{12} is seen in pernicious anemia. If repetition of the test with addition of IF results in increased radioactivity in the urine, lack of functional IF is confirmed. Decreased glomerular filtration, due to either renal disease or aging, and improper urine collection invalidate the test results. With elderly patients, collection and evaluation of a 48-hour urine specimen will improve the accuracy of the test.

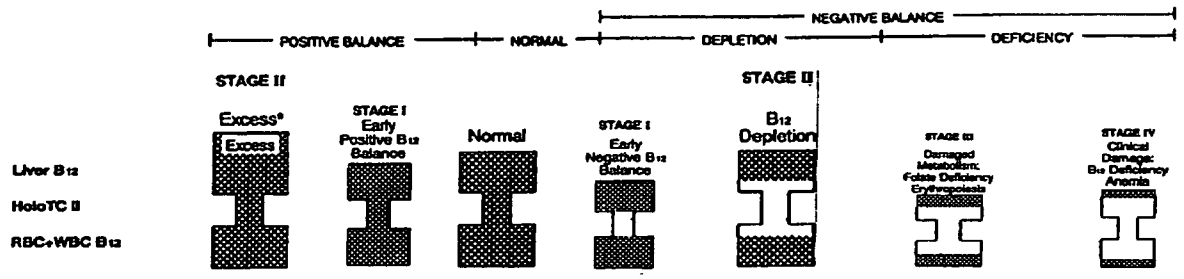
A recent study evaluated the effect of light on serum B_{12} concentrations (111–812 ng/L). Under typical storage conditions encountered in a clinical laboratory, B_{12} was not affected by light for up to 24 hours after collection when stored at room temperature (20–25°C).[154] Depending on the assessment method employed, serum levels of B_{12} range from approximately 200 to 900 pg/mL.[112] B_{12}-deficient erythropoiesis is associated with levels less than 100 pg/mL (74 pmol/L).[66,95] Serum folate and vitamin B_{12} levels must be determined in patients with megaloblastic anemia to pinpoint its etiology. Large-dose folate therapy may bring about transient improvement of megaloblastic anemia associated with B_{12} deficiency, but neurologic damage will develop or progress, often irreversibly. It is essential to distinguish the true nature of the underlying disorder (*e.g.*, folate or B_{12} deficiency) so that appropriate therapy may be provided as quickly as possible.

Some patients with serum B_{12} in the lower portion of the reference range may still develop PA. B_{12} deficiency may be by assessment of serum methylmalonate and homocysteine concentrations (Table 29-20).[95,96,168,245,246] Elevated levels of methylmalonic acid (MMA) and total homocysteine are detected in over 90% of cases of cobalamin deficiency. Measurement of urinary MMA excretion is also diagnostically useful. Increase in these metabolites often occurs before any other clinical evidence of deficiency is manifested. Serum MMA levels >950 nmol/L (110–950 nmol/L) and total homocysteine concentrations >29 micromoles/L (6–29 micromoles/L) indicate B_{12} deficiency even in the presence of normal hematologic parameters. An automated assay of MMA in serum and urine by derivatization with 1-pyrenyldiazomethane, liquid chromatography, and fluorescence detection has recently been described.[233] The risk factor for occlusive atherosclerosis is increased by hyperhomocysteinemia. Improved vitamin B_{12} status normalizes homocysteine levels within weeks, thereby reducing the patient's risk of coronary artery disease.

Cobalamin determinations may be by microbiologic or radioligand assays. Although a variety of vitamin B_{12}–dependent test organisms have been used, including *Euglena gracilis, Lactobacillus leichmannii* (ATCC 7830) remains the microorganism of choice.[79] Microbiological assay is used as the reference method or in a research setting. In the clinical laboratory, radioassays are routinely used for determination of serum B_{12} levels. Differential radioassays measure cobalamin content more accurately than do microbiologic assays, since noncobalamin corrinoids not utilized by humans will support microbial growth.[95] Plasma transcobalamins must be heat denatured (boiling) or subjected to alkaline pH inactivation (no-boil) prior to either microbiologic or radioassay of the specimen to release the cobalamin for measurement.

Radioisotope dilution methods are the most widely used assays for cobalamin. These competitive inhibition radioassays measure the extent to which cobalamin, after being freed from bound materials, competes with radioactive cyanocobalamin for binding sites on a protein.[66] Radioligand assays may be either RIA or CBP procedures. In the case of CBP assays, purified IF has been strongly recommended as the cobalamin-binding protein. A semiautomated radioassay system makes possible simultaneous assessments of serum B_{12} and folate, following off-line denaturation of endogenous binding proteins.[45] Purified IF is used as the competitive binding protein, with solid-phase adsorbent separating free and bound ^{57}Co. Recently, it has been reported that no boiling or other pretreatment of patient specimen is required when a non-intrinsic factor blocking agent is used along with a magnetizable solid-phase separation system.[111] This assay is highly specific for cobalamin. With elimination of a pretreatment requirement, and ease of separation

TABLE 29-20. Sequential Stages of Vitamin B-12 Status. Biochemical and Hematological Sequence of Events as Negative Vitamin B-12 Balance Progresses. [© 1990, 1993 Victor Herbert (Modified 1993 to Include Homocysteine).]



HoloTC II (pg/ml) (in equilibrium with TCII receptors [on DNA-synthesizing cells])	>100	>100	>50	<40	<40	<40	<40
TC II % sat. (Caution: Apo TCII is an acute phase reactant)	>5%	>5%	>5%	<4%	<4%	<4%	<4%
Holohap (pg/ml)¥ (in equilibrium with haptocorrin receptors [on B_{12}-storage cells])	>500	>400	>180	>180	<150 ¥	<100	<100
dU Suppression	Normal	Normal	Normal	Normal	Normal	Abnormal	Abnormal
Hypersegmentation	No	No	No	No	No	Yes	Yes
TBBC† % sat.	>50%	>40	>15%	>15%	>15%	<15%	<10%
Hap % sat.	>50%	>40	>20%	>20%	>20%	<20%	<10%
RBC Folate (ng/mL)	>160	>160	>160	>160	>160	<140	<100
RBC Cobalamin (ng/ml)	<800	<600	300–800	<300	<200	<150	<100
Homocystiene †	No	No	No	No	No	Yes	No
Erythrocytes	Normal	Normal	Normal	Normal	Normal	Normal	Macroovalocytic
MCV	Normal	Normal	Normal	Normal	Normal	Normal	Elevated
Hemoglobin	Normal	Normal	Normal	Normal	Normal	Normal	Low
TC II	Normal	Normal	Normal	Normal	Normal	Elevated	Elevated
Homocysteine and/or Methylmalonate †≠	No	No	No	No	No	?	Yes
Myelin Damage	No*	No	No	No	No	?	Frequent
Holo TC II cell receptors	Normal	Normal	Normal	Up-regulated?	Down-regulated?	Elevated in plasma	

**Cyanocobalamin excesses (injected or intranasal) produce transient rise in B_{12} analogues on B_{12} delivery protein (TC II); the significance of such rises is unknown (Herbert et al., 1987). Cyanocobalamin acts as an anti-B_{12} in a rare congenital defect in B_{12} metabolism.*

≠ In serum and urine.

† TBBC = Total B_{12} binding capacity.

¥ Low holohaptocorrin correlates with liver cell B_{12} depletion. There may be hematopoietic cell and glial cell B_{12} depletion prior to liver cell depletion, and those cells may be in STAGE III or IV negative B_{12} balance while liver cells are still in STAGE II.

achieved in a magnetic radioassay, a fully automated continuous-flow procedure can be realized. Assay automation of B_{12} on the Abbott IM$_x$ provides rapid results in a nonradioisotopic format.[129] B_{12} deficiency can be detected and quantitated by measuring methylmalonic acid in urine or assessing its serum level using capillary gas chromatography/mass spectrometry.[245,246]

SUMMARY

The Joint Commission on Accreditation of Healthcare Organizations (JCAHO) is mandating more stringent nutritional review of all patients. There can be no doubt that this will impact the clinical laboratory. The clinical laboratorian will be required to know more about vitamins, their biochemical functions and physiologic roles, and the best assay methodologies to use to provide the clinician with timely information on the patient's nutritional status. There are financial implications to optimizing a patient's nutritional status, thereby hastening the desired medical outcomes and reducing the patient's length of stay in the hospital.

The general public is also increasingly concerned with health promotion and disease prevention. Supplemental use of vitamins to increase longevity and improve the quality of life is regularly advocated in the media. Vitamin sales is a multi-billion-dollar commercial enterprise in this country. While there is strong support for the beneficial effects of vitamins in the prevention of certain cancers and cardiovascular disease, there is also concern over the possibility of toxicity from overly aggressive vitamin supplementation.[200]

Historically, medicine has focused more attention on conditions of vitamin deficiency than excess. Despite the high standard of living in this country, significant numbers of individuals are characterized by an overall vitamin status that is suboptimal or overtly deficient. Nutritional requirements in special physiological states such as growth, preg-

nancy and lactation, and aging may not be met by dietary consumption. For example, age-related changes in vitamin status due to altered dietary practices, physiologic changes, and drug-nutrient interaction contribute to the risk for deficiency of one or more vitamins among the 32 million Americans who are over 65 years of age.

Biochemical determinations of vitamin status and the monitoring of nutritional support will increase in the years ahead. In the future, vitamin assays will not be viewed as esoteric reference laboratory procedures; rather they will be acknowledged as essential for the promotion of wellness and for the cost-effective provision of quality health care.

CASE STUDY 29-1

During her most recent physical examination, a blood pressure of 175/96 had been recorded for a widowed, 65-year-old female. Over the past 3 years, her blood pressure as recorded on annual physical examinations had gradually risen, but this report was the first clear indication of hypertension. Her physician prescribed 150 mg of hydralazine per day, administered orally. In follow-up office visits, her physician noted that the dosage prescribed was not producing a satisfactory lowering of the patient's blood pressure. Adjustment of dosage was attempted, and satisfactory results were finally achieved with 400 mg of hydralazine daily. Several months after initiation of therapy, the patient's daughter called the physician to report pronounced changes in her mother's personality. Her usual optimism had been replaced by depression and irritability. In addition, her daughter indicated that the woman no longer appeared interested in her house or her family. She was reluctant to cook for herself but had purchased a supply of high-protein supplement, which she consumed for nourishment. Such a lack of responsibility was not in keeping with her mother's traditional behavior. These changes, coupled with the appearance of a rash on her mother's forehead, prompted the daughter to bring her mother to the clinic. Upon review of the medication record and noting signs of peripheral nerve inflammation in the patient, the physician requested the laboratory to evaluate the patient's vitamin B_6 status.

Questions

1. What type of assessment procedure will the laboratory be most likely to employ in evaluating the patient's vitamin B_6 status?
2. Identify the patient specimen required for testing and any special precautions to be taken in its handling or processing.
3. What clinical manifestations suggested a vitamin B_6 deficiency to the physician?
4. In what way is it likely that the patient's medication and dietary practices contributed to development of a B_6 deficiency?
5. A marginal or deficient vitamin B_6 status is indicated by laboratory values of what magnitude?

CASE STUDY 29-2

A 62-year-old male had been admitted to the hospital with a diagnosis of acute myocardial infarction. Anticoagulant therapy was initiated in an attempt to reduce the incidence of secondary thromboembolism. While he was hospitalized, heparin therapy had been initiated, and, upon discharge, the patient was switched to Coumadin. For 3 months after leaving the hospital, the patient had been completely stable on a Coumadin regimen of 30 mg per week. During a follow-up visit to his physician, the man's prothrombin time was reported as 12 seconds as compared with previously obtained PT times of 22 to 24 seconds. Effective oral anticoagulant therapy calls for maintenance of a prothrombin time that exceeds "normal" by 1.5 to 1.7 times. Review of the patient's medication record did not suggest drug interference as the basis for the decreased anticoagulant effect. A careful dietary history provided an explanation for the newly acquired warfarin resistance.

Questions

1. Excessive intake of what vitamin is likely to account for the observed shortening of prothrombin time?
2. Describe the physiologic function of this vitamin.
3. Suggest possible dietary practices that could induce warfarin (Coumadin) resistance.

Eli Lilly and Company Limited

Lilly House
Priestley Road
Basingstoke
Hampshire
RG24 9NL

Telephone:	**+44 (0)1256 315 000**
Facsimile:	**+44 (0)1256 775 858**
WWW:	**http://www.lilly.co.uk**
Customer Care direct line:	**+44 (0)1256 315 999**
Medical Information e-mail:	**ukmedinfo@lilly.com**
Medical Information facsimile:	**+44 (0)1256 775 569**

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Alimta 500mg powder for concentrate for solution for infusion

Table of Contents

1. NAME OF THE MEDICINAL PRODUCT

Alimta*▼ 500mg powder for concentrate for solution for infusion.

2. QUALITATIVE AND QUANTITATIVE COMPOSITION

Each vial contains 500mg of pemetrexed (as pemetrexed disodium).
Each vial must be reconstituted with 20ml of sodium chloride 9mg/ml (0.9%) solution for injection resulting in 25mg/ml of solution. The appropriate volume of required dose is removed from the vial and further diluted to 100ml with sodium chloride 9mg/ml (0.9%) solution for injection (see section 6.6).
Excipients: For a full list of excipients see section 6.1.

3. PHARMACEUTICAL FORM

Powder for concentrate for solution for infusion.
A white to either light yellow or green-yellow lyophilised powder.

4. CLINICAL PARTICULARS

4.1 Therapeutic indications

Alimta in combination with cisplatin is indicated for the treatment of chemotherapy naive patients with unresectable malignant pleural mesothelioma.
Alimta is indicated as monotherapy for the treatment of patients with locally advanced or metastatic non-small cell lung cancer after prior chemotherapy.

4.2 Posology and method of administration

Alimta must only be administered under the supervision of a physician qualified in the use of anti-cancer chemotherapy.
The Alimta solution must be prepared according to the instructions provided in section 6.6.

Malignant Pleural Mesothelioma

In patients treated for malignant pleural mesothelioma, the recommended dose of Alimta is 500mg/m^2 of body surface area (BSA) administered as an intravenous infusion over 10 minutes on the first day of each 21-day cycle. The recommended dose of cisplatin is 75mg/m^2 BSA infused over two hours approximately 30 minutes after completion of the pemetrexed infusion on the first day of each 21-day cycle. Patients must receive adequate anti-emetic treatment and appropriate hydration prior to and/or after receiving cisplatin (see also cisplatin Summary of Product Characteristics for specific dosing advice).

Non-Small Cell Lung Cancer

In patients treated for non-small cell lung cancer, the recommended dose of Alimta is 500mg/m^2 BSA administered as an intravenous infusion over 10 minutes on the first day of each 21-day cycle.

Pre-Medication Regimen

To reduce the incidence and severity of skin reactions, a corticosteroid should be given the day prior to, on the day of, and the day after pemetrexed administration. The corticosteroid should be equivalent to 4mg of dexamethasone administered orally twice a day (see section 4.4).
To reduce toxicity, patients treated with pemetrexed must also receive vitamin supplementation (see section 4.4). Patients must take oral folic acid or a multivitamin containing folic acid (350 to 1,000 micrograms) on a daily basis. At least five doses of folic acid must be taken during the seven days preceding the first dose of pemetrexed, and dosing must continue during the full course of therapy and for 21 days after the last dose of pemetrexed. Patients must also receive an intramuscular injection of vitamin B_{12} (1,000 micrograms) in the week preceding the first dose of pemetrexed and once every three cycles thereafter. Subsequent vitamin B_{12} injections may be given on the same day as pemetrexed.

Monitoring

Patients receiving pemetrexed should be monitored before each dose with a complete blood count, including a differential white cell count (WCC) and platelet count. Prior to each chemotherapy administration, blood chemistry tests should be collected to evaluate renal and hepatic function. Before the start of any cycle of chemotherapy, patients are required to have the following: absolute neutrophil count (ANC) should be ≥1,500 cells/mm^3 and platelets should be ≥ 100,000 cells/mm^3.
Creatinine clearance should be ≥45ml/min.
The total bilirubin should be ≤1.5-times upper limit of normal. Alkaline phosphatase (AP), aspartate transaminase (AST or SGOT), and alanine transaminase (ALT or SGPT) should be ≤3-times upper limit of normal. Alkaline phosphatase, AST, and ALT ≤5-times upper limit of normal is acceptable if liver has tumour involvement.

Dose Adjustments

Dose adjustments at the start of a subsequent cycle should be based on nadir haematologic counts or maximum non-haematologic toxicity from the preceding cycle of therapy. Treatment may be delayed to allow sufficient time for recovery. Upon recovery, patients should be retreated using the guidelines in *Tables 1, 2, and 3*, which are applicable for Alimta used as a single-agent or in combination with cisplatin.
Table 1. **Dose Modification Table for Alimta (as Single-Agent or in Combination) and Cisplatin - Haematologic Toxicities**

Nadir ANC <500/mm^3 and nadir platelets ≥ 50,000/mm^3	75% of previous dose (both Alimta and cisplatin)
Nadir platelets <50,000/mm^3 regardless of nadir ANC	50% of previous dose (both Alimta and cisplatin)

If patients develop non-haematologic toxicities ≥Grade 3 (excluding neurotoxicity), Alimta should be withheld until resolution to less than or equal to the patient's pre-therapy value. Treatment should be resumed according to the guidelines in *Table 2*.
Table 2. **Dose Modification Table for Alimta (as Single-Agent or in Combination) and Cisplatin - Non-Haematologic Toxicities**[a, b]

	Dose of Alimta (mg/m^2)	Dose for Cisplatin (mg/m^2)
Any Grade 3 or 4 toxicities except mucositis	75% of previous dose	75% of previous dose
Any diarrhoea requiring hospitalisation (irrespective of grade) or Grade 3 or 4 diarrhoea	75% of previous dose	75% of previous dose
Grade 3 or 4 mucositis	50% of previous dose	100% of previous dose
[a] National Cancer Institute Common Toxicity Criteria (CTC). [b] Excluding neurotoxicity.		

In the event of neurotoxicity, the recommended dose adjustment for Alimta and cisplatin is documented in *Table 3*. Patients should discontinue therapy if Grade 3 or 4 neurotoxicity is observed.

Table 3. **Dose Modification Table for Alimta (as Single-Agent or in Combination) and Cisplatin – Neurotoxicity**

CTC* Grade	Dose of Alimta (mg/m^2)	Dose for Cisplatin (mg/m^2)
0-1	100% of previous dose	100% of previous dose
2	100% of previous dose	50% of previous dose
*National Cancer Institute Common Toxicity Criteria (CTC).		

Treatment with Alimta should be discontinued if a patient experiences any haematologic or non-haematologic Grade 3 or 4 toxicity after 2 dose reductions or immediately if Grade 3 or 4 neurotoxicity is observed.

Elderly: In clinical studies, there has been no indication that patients 65 years of age or older are at increased risk of adverse events compared to patients younger than 65 years old. No dose reductions other than those recommended for all patients are necessary.

Children and adolescents: Alimta is not recommended for use in children below 18 years of age due to insufficient data on safety and efficacy.

Patients with renal impairment (standard Cockcroft and Gault formula or glomerular filtration rate measured Tc99m-DPTA serum clearance method): Pemetrexed is primarily eliminated unchanged by renal excretion. In clinical studies, patients with creatinine clearance of ≥45ml/min required no dose adjustments other than those recommended for all patients. There are insufficient data on the use of pemetrexed in patients with creatinine clearance below 45ml/min; therefore, the use of pemetrexed is not recommended (see section 4.4).

Patients with hepatic impairment: No relationships between AST (SGOT), ALT (SGPT), or total bilirubin and pemetrexed pharmacokinetics were identified. However, patients with hepatic impairment, such as bilirubin >1.5-times the upper limit of normal and/or transaminase >3.0-times the upper limit of normal (hepatic metastases absent) or >5.0-times the upper limit of normal (hepatic metastases present), have not been specifically studied.

4.3 Contraindications

Hypersensitivity to the active substance or to any of the excipients.
Breast-feeding must be discontinued during pemetrexed therapy (see section 4.6).
Concomitant yellow fever vaccine (see section 4.5).

4.4 Special warnings and precautions for use

Pemetrexed can suppress bone marrow function as manifested by neutropenia, thrombocytopenia, and anaemia (or pancytopenia) (see section 4.8). Myelosuppression is usually the dose-limiting toxicity. Patients should be monitored for myelosuppression during therapy and pemetrexed should not be given to patients until absolute neutrophil count (ANC) returns to ≥ 1500 cells/mm^3 and platelet count returns to ≥100,000 cells/mm^3. Dose reductions for subsequent cycles are based on nadir ANC, platelet count, and maximum non-haematologic toxicity seen from the previous cycle (see section 4.2).

In the Phase 3 mesothelioma trial, overall less toxicity and reduction in Grade 3/4 haematologic and non-haematologic toxicities, such as neutropenia, febrile neutropenia, and infection with Grade 3/4 neutropenia, were reported when pre-treatment with folic acid and vitamin B_{12} was administered. Therefore, patients treated with pemetrexed must be instructed to take folic acid and vitamin B_{12} as a prophylactic measure to reduce treatment-related toxicity (see section 4.2).

Skin reactions have been reported in patients not pre-treated with a corticosteroid. Pre-treatment with dexamethasone (or equivalent) can reduce the incidence and severity of skin reactions (see section 4.2).

An insufficient number of patients has been studied with creatinine clearance of below 45ml/min. Therefore, the use of pemetrexed in patients with creatinine clearance of <45ml/min is not recommended (see section 4.2).

Patients with mild to moderate renal insufficiency (creatinine clearance from 45 to 79ml/min) should avoid taking non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, and aspirin (>1.3g daily) for 2 days before, on the day of, and 2 days following pemetrexed administration (see section 4.5). All patients eligible for pemetrexed therapy should avoid taking NSAIDs with long elimination half-lives for at least 5 days prior to, on the day, and at least 2 days following pemetrexed administration (see section 4.5).

Serious renal events, including acute renal failure, have been reported with pemetrexed alone or in association with other chemotherapeutic agents. Many of the patients in whom these occurred had underlying risk factors for the development of renal events, including dehydration or pre-existing hypertension or diabetes.

The effect of third space fluid, such as pleural effusion or ascites, on pemetrexed is unknown. In patients with clinically significant third space fluid, consideration should be given to draining the effusion prior to pemetrexed administration.

Due to the gastro-intestinal toxicity of pemetrexed given in combination with cisplatin, severe dehydration has been observed. Therefore, patients should receive adequate anti-emetic treatment and appropriate hydration prior to and/or after receiving treatment.

Serious cardiovascular events, including myocardial infarction and cerebrovascular events, have been uncommonly reported during clinical studies with pemetrexed, usually when given in combination with another cytotoxic agent. Most of the patients in whom these events have been observed had pre-existing cardiovascular risk factors (see section 4.8).

Immunodepressed status is common in cancer patients. As a result, concomitant use of live attenuated vaccines (except yellow fever, which is contra-indicated) is not recommended (see section 4.3 and section 4.5).

Pemetrexed can have genetically damaging effects. Sexually mature males are advised not to father a child during the treatment and up to 6 months thereafter. Contraceptive measures or abstinence are recommended. Owing to the possibility of pemetrexed treatment causing irreversible infertility, men are advised to seek counselling on sperm storage before starting treatment.

Women of childbearing potential must use effective contraception during treatment with pemetrexed (see section 4.6).

4.5 Interaction with other medicinal products and other forms of interaction

Pemetrexed is mainly eliminated unchanged renally by tubular secretion and to a lesser extent by glomerular filtration. Concomitant administration of nephrotoxic drugs (eg, aminoglycoside, loop diuretics, platinum compounds, cyclosporin) could potentially result in delayed clearance of pemetrexed. This combination should be used with caution. If necessary, creatinine clearance should be closely monitored.
Concomitant administration of substances that are also tubularly secreted (eg, probenecid, penicillin) could potentially result in delayed clearance of pemetrexed. Caution should be made when these drugs are combined with pemetrexed. If necessary, creatinine clearance should be closely monitored.
In patients with normal renal function (creatinine clearance ≥80ml/min), high doses of non-steroidal anti-inflammatory drugs (NSAIDs, such as ibuprofen >1600mg/day) and aspirin at higher dosage (≥1.3g daily) may decrease pemetrexed elimination and, consequently, increase the occurrence of pemetrexed adverse events. Therefore, caution should be made when administering higher doses of NSAIDs or aspirin at higher dosage concurrently with pemetrexed to patients with normal function (creatinine clearance ≥80ml/min).
In patients with mild to moderate renal insufficiency (creatinine clearance from 45 to 79ml/min), the concomitant administration of pemetrexed with NSAIDs (eg, ibuprofen) or aspirin at higher dosage should be avoided for 2 days before, on the day of, and 2 days following pemetrexed administration (see section 4.4).
In the absence of data regarding potential interaction with NSAIDs having longer half-lives, such as piroxicam or rofecoxib, the concomitant administration with pemetrexed should be avoided for at least 5 days prior to, on the day, and at least 2 days following pemetrexed administration (see section 4.4).
Pemetrexed undergoes limited hepatic metabolism. Results from *in vitro* studies with human liver microsomes indicated that pemetrexed would not be predicted to cause clinically significant inhibition of the metabolic clearance of drugs metabolised by CYP3A, CYP2D6, CYP2C9, and CYP1A2.

Interactions Common to all Cytotoxics

Due to the increased thrombotic risk in patients with cancer, the use of anticoagulation treatment is frequent. The high intra-individual variability of the coagulation status during diseases and the possibility of interaction between oral anticoagulants and anti-cancer chemotherapy require increased frequency of INR (International Normalised Ratio) monitoring, if it is decided to treat the patient with oral anticoagulants.

Concomitant Use Contra-Indicated

Yellow fever vaccine: Risk of fatal generalised vaccinale disease (see section 4.3).

Concomitant Use Not Recommended

Live attenuated vaccines (except yellow fever): Risk of systemic, possibly fatal, disease. The risk is increased in subjects who are already immunosuppressed by their underlying disease. Use an inactivated vaccine where it exists (poliomyelitis) (see section 4.4).

4.6 Pregnancy and lactation

There are no data from the use of pemetrexed in pregnant women but pemetrexed, like other anti-metabolites, is suspected to cause serious birth defects when administered during pregnancy. Animal studies have shown reproductive toxicity (see section 5.3). Pemetrexed should not be used during pregnancy unless clearly necessary, after a careful consideration of the needs of the mother and the risk for the foetus (see section 4.4).

Women of childbearing potential must use effective contraception during treatment with pemetrexed. Pemetrexed can have genetically damaging effects. Sexually mature males are advised not to father a child during the treatment and up to 6 months thereafter. Contraceptive measures or abstinence are recommended. Owing to the possibility of pemetrexed treatment causing irreversible infertility, men are advised to seek counselling on sperm storage before starting treatment.
It is not known whether pemetrexed is excreted in human milk and adverse reactions on the suckling child cannot be excluded. Breast-feeding must be discontinued during pemetrexed therapy (see section 4.3).

4.7 Effects on ability to drive and use machines

No studies on the effects on the ability to drive and use machines have been performed. However, it has been reported that pemetrexed may cause fatigue. Therefore, patients should be cautioned against driving or operating machines if this event occurs.

4.8 Undesirable effects

The table below provides the frequency and severity of undesirable effects that have been reported in >5% of 168 patients with mesothelioma who were randomised to receive cisplatin and pemetrexed and 163 patients with mesothelioma randomised to receive single-agent cisplatin. In both treatment arms, these chemonaive patients were fully supplemented with folic acid and vitamin B_{12}.

Adverse Reactions

Frequency estimate: Very common (≥1/10), common (≥1/100 and <1/10), uncommon (≥1/1,000 and <1/100), rare (≥1/10,000 and <1/1,000), very rare (<1/10,000) and not known (cannot be estimated from available data – spontaneous reports).
Within each frequency grouping, undesirable effects are presented in order of decreasing seriousness.

System Organ Class	**Frequency**	**Event***	**Pemetrexed/Cisplatin (n = 168)**		**Cisplatin (n = 163)**	
			All Grades Toxicity (%)	**Grade 3-4 Toxicity (%)**	**All Grades Toxicity (%)**	**Grade 3-4 Toxicity (%)**
Blood and lymphatic system disorders	Very common	Neutrophils/granulocytes decreased	56.0	23.2	13.5	3.1
		Leucocytes decreased	53.0	14.9	16.6	0.6
		Haemoglobin decreased	26.2	4.2	10.4	0.0
		Platelets decreased	23.2	5.4	8.6	0.0
Eye disorders	Common	Conjunctivitis	5.4	0.0	0.6	0.0
Gastro-	Very	Diarrhoea	16.7	3.6	8.0	0.0

intestinal disorders	common	Vomiting	56.5	10.7	49.7	4.3
		Stomatitis/pharyngitis	23.2	3.0	6.1	0.0
		Nausea	82.1	11.9	76.7	5.5
		Anorexia	20.2	1.2	14.1	0.6
		Constipation	11.9	0.6	7.4	0.6
	Common	Dyspepsia	5.4	0.6	0.6	0.0
General disorders	Very common	Fatigue	47.6	10.1	42.3	9.2
Metabolism and nutrition disorders	Common	Dehydration	6.5	4.2	0.6	0.6
Nervous system disorders	Very common	Neuropathy - sensory	10.1	0.0	9.8	0.6
	Common	Dysgeusia	7.7	0.0	6.1	0.0
Renal and urinary disorders	Very common	Creatinine elevation	10.7	0.6	9.8	1.2
		Creatinine clearance decreased**	16.1	0.6	17.8	1.8
Skin and subcutaneous tissue disorders	Very common	Rash	16.1	0.6	4.9	0.0
		Alopecia	11.3	0.0	5.5	0.0

*Refer to National Cancer Institute CTC version 2 for each grade of toxicity, except the term "creatinine clearance decreased"** which is derived from the term "renal/genitourinary other".

Very common - ≥10%; common is normally defined as >1% and <10%. For the purpose of this table, a cut-off of 5% was used for inclusion of all events where the reporter considered a possible relationship to pemetrexed and cisplatin.

Clinically relevant CTC toxicities that were reported in >1% and ≤ 5% (common) of the patients that were randomly assigned to receive cisplatin and pemetrexed include: renal failure, infection, pyrexia, febrile neutropenia, increased AST, ALT, and GGT, urticaria, and chest pain.
Clinically relevant CTC toxicities that were reported in ≤ 1% of the patients that were randomly assigned to receive cisplatin and pemetrexed include arrhythmia and motor neuropathy.
The table below provides the frequency and severity of undesirable effects that have been reported in >5% of 265 patients randomly assigned to receive single-agent pemetrexed with folic acid and vitamin B_{12} supplementation and 276 patients randomly assigned to receive single-agent docetaxel. All patients were diagnosed with locally advanced or metastatic non-small cell lung cancer and received prior chemotherapy.

System Organ Class	**Frequency**	**Event***	**Pemetrexed n = 265**		**Docetaxel n = 276**	
			All Grades Toxicity (%)	**Grade 3-4 Toxicity (%)**	**All Grades Toxicity (%)**	**Grade 3-4 Toxicity (%)**

Blood and lymphatic system disorders	Very common	Neutrophils/granulocytes decreased	10.9	5.3	45.3	40.2
		Leucocytes decreased	12.1	4.2	34.1	27.2
		Haemoglobin decreased	19.2	4.2	22.1	4.3
	Common	Platelets decreased	8.3	1.9	1.1	0.4
Gastro-intestinal disorders	Very common	Diarrhoea	12.8	0.4	24.3	2.5
		Vomiting	16.2	1.5	12.0	1.1
		Stomatitis/pharyngitis	14.7	1.1	17.4	1.1
		Nausea	30.9	2.6	16.7	1.8
		Anorexia	21.9	1.9	23.9	2.5
	Common	Constipation	5.7	0.0	4.0	0.0
General disorders	Very common	Fatigue	34.0	5.3	35.9	5.4
	Common	Fever	8.3	0.0	7.6	0.0
Hepatobiliary disorders	Common	SGPT (ALT) elevation	7.9	1.9	1.4	0.0
		SGOT (AST) elevation	6.8	1.1	0.7	0.0
Skin and subcutaneous tissue disorders	Very common	Rash/desquamation	14.0	0.0	6.2	0.0
	Common	Pruritus	6.8	0.4	1.8	0.0
		Alopecia	6.4	0.4	37.7	2.2

*Refer to National Cancer Institute CTC version 2 for each grade of toxicity.

Very common - ≥10%; common is normally defined as >1% and <10%. For the purpose of this table, a cut-off of 5% was used for inclusion of all events where the reporter considered a possible relationship to pemetrexed.

) Clinically relevant CTC toxicities that were reported in >1% and ≤5% (common) of the patients that were randomly assigned to pemetrexed include: infection without neutropenia, febrile neutropenia, allergic reaction/hypersensitivity, increased creatinine, motor neuropathy, sensory neuropathy, erythema multiforme, and abdominal pain.

Clinically relevant CTC toxicities that were reported in ≤1% of the patients that were randomly assigned to pemetrexed include supraventricular arrhythmias.

Clinically relevant Grade 3 and Grade 4 laboratory toxicities were similar between integrated Phase 2 results from three single-agent pemetrexed studies (n = 164) and the Phase 3 single-agent pemetrexed study described above, with the exception of neutropenia (12.8% versus 5.3%, respectively) and alanine transaminase elevation (15.2% versus 1.9%, respectively). These differences were likely due to differences in the patient population, since the Phase 2 studies included both chemonaive and heavily pre-treated breast cancer patients with pre-existing liver metastases and/or abnormal baseline liver function tests.

Serious cardiovascular and cerebrovascular events, including myocardial infarction, angina pectoris, cerebrovascular accident, and transient ischaemic attack, have been uncommonly reported during clinical studies with pemetrexed, usually when given in combination with another cytotoxic agent. Most of the patients in whom these events have been observed had pre-existing cardiovascular risk factors.

Rare cases of hepatitis, potentially serious, have been reported during clinical studies with

pemetrexed.
Pancytopenia has been uncommonly reported during clinical trials with pemetrexed.
During post-marketing surveillance, the following adverse reactions have been reported in patients treated with pemetrexed:
Rare cases of colitis have been reported in patients treated with pemetrexed.
Cases of acute renal failure have been reported with pemetrexed alone or in association with other chemotherapeutic agents (see section 4.4).

4.9 Overdose

Reported symptoms of overdose include neutropenia, anaemia, thrombocytopenia, mucositis, sensory polyneuropathy, and rash. Anticipated complications of overdose include bone marrow suppression as manifested by neutropenia, thrombocytopenia, and anaemia. In addition, infection with or without fever, diarrhoea, and/or mucositis may be seen. In the event of suspected overdose, patients should be monitored with blood counts and should receive supportive therapy as necessary. The use of calcium folinate/folinic acid in the management of pemetrexed overdose should be considered.

5. PHARMACOLOGICAL PROPERTIES

5.1 Pharmacodynamic properties

Pharmacotherapeutic group: Folic acid analogues. *ATC code:* L01BA04.
Alimta is a multi-targeted anti-cancer antifolate agent that exerts its action by disrupting crucial folate-dependent metabolic processes essential for cell replication.
In vitro studies have shown that pemetrexed behaves as a multi-targeted antifolate by inhibiting thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT), which are key folate-dependent enzymes for the *de novo* biosynthesis of thymidine and purine nucleotides. Pemetrexed is transported into cells by both the reduced folate carrier and membrane folate binding protein transport systems. Once in the cell, pemetrexed is rapidly and efficiently converted to polyglutamate forms by the enzyme folylpolyglutamate synthetase. The polyglutamate forms are retained in cells and are even more potent inhibitors of TS and GARFT. Polyglutamation is a time- and concentration-dependent process that occurs in tumour cells and, to a lesser extent, in normal tissues. Polyglutamated metabolites have an increased intracellular half-life resulting in prolonged drug action in malignant cells.

Clinical Efficacy

EMPHACIS, a multi-centre, randomised, single-blind Phase 3 study of Alimta plus cisplatin versus cisplatin in chemonaive patients with malignant pleural mesothelioma, has shown that patients treated with Alimta and cisplatin had a clinically meaningful 2.8-month median survival advantage over patients receiving cisplatin alone.
During the study, low-dose folic acid and vitamin B_{12} supplementation was introduced to patients' therapy to reduce toxicity. The primary analysis of this study was performed on the population of all patients randomly assigned to a treatment arm who received study drug (randomised and treated). A subgroup analysis was performed on patients who received folic acid and vitamin B_{12} supplementation during the entire course of study therapy (fully supplemented). The results of

these analyses of efficacy are summarised in the table below.

Efficacy of Alimta Plus Cisplatin vs Cisplatin in Malignant Pleural Mesothelioma

	Randomised and Treated Patients		**Fully Supplemented Patients**	
Efficacy Parameter	Alimta/Cisplatin (n = 226)	Cisplatin (n = 222)	Alimta/Cisplatin (n = 168)	Cisplatin (n = 163)
Median overall survival (months)	12.1	9.3	13.3	10.0
(95% CI)	(10.0-14.4)	(7.8-10.7)	(11.4-14.9)	(8.4-11.9)
Log rank *P* -value*	0.020		0.051	
Median time to tumour progression (months)	5.7	3.9	6.1	3.9
(95% CI)	(4.9-6.5)	(2.8-4.4)	(5.3-7.0)	(2.8-4.5)
Log rank *P* -value*	0.001		0.008	
Time to treatment failure (months)	4.5	2.7	4.7	2.7
(95% CI)	(3.9-4.9)	(2.1-2.9)	(4.3-5.6)	(2.2-3.1)
Log rank *P* -value*	0.001		0.001	
Overall response rate**	41.3%	16.7%	45.5%	19.6%
(95% CI)	(34.8-48.1)	(12.0-22.2)	(37.8-53.4)	(13.8-26.6)
Fisher's exact *P* -value*	<0.001		<0.001	

Abbreviation: CI = confidence interval.

**P* -value refers to comparison between arms.

**In the Alimta/cisplatin arm, randomised and treated (n = 225) and fully supplemented (n = 167).

A statistically significant improvement of the clinically relevant symptoms (pain and dyspnoea) associated with malignant pleural mesothelioma in the Alimta/cisplatin arm (212 patients) versus the cisplatin arm alone (218 patients) was demonstrated using the Lung Cancer Symptom Scale. Statistically significant differences in pulmonary function tests were also observed. The separation between the treatment arms was achieved by improvement in lung function in the Alimta/cisplatin arm and deterioration of lung function over time in the control arm.
There are limited data in patients with malignant pleural mesothelioma treated with Alimta alone. Alimta at a dose of 500mg/m^2 was studied as a single-agent in 64 chemonaive patients with malignant pleural mesothelioma. The overall response rate was 14.1%.
A multi-centre, randomised, open-label Phase 3 study of Alimta versus docetaxel in patients with locally advanced or metastatic NSCLC after prior chemotherapy has shown median survival times of 8.3 months for patients treated with Alimta (intent to treat population n = 283) and 7.9 months for patients treated with docetaxel (ITT n = 288).

Efficacy of Alimta vs Docetaxel in NSCLC - ITT Population

	Alimta	**Docetaxel**
Survival time (months)	(n = 283)	(n = 288)
• Median (m)	8.3	7.9
• 95% CI for median	(7.0-9.4)	(6.3-9.2)
• HR	0.99	
• 95% CI for HR	(.82-1.20)	
• Non-inferiority *P* -value (HR)	.226	
Progression free survival (months)	(n = 283)	(n = 288)
• Median	2.9	2.9
• HR (95% CI)	0.97 (.82-1.16)	
Time to treatment failure (TTTF - months)	(n = 283)	(n = 288)
• Median	2.3	2.1
• HR (95% CI)	0.84 (.71-.997)	
Response (n: qualified for response)	(n = 264)	(n = 274)
• Response rate (%) (95% CI)	9.1 (5.9-13.2)	8.8 (5.7-12.8)
• Stable disease (%)	45.8	46.4
Abbreviations: CI = confidence interval; HR = hazard ratio; ITT = intent to treat; n = total population size.		

5.2 Pharmacokinetic properties

The pharmacokinetic properties of pemetrexed following single-agent administration have been evaluated in 426 cancer patients with a variety of solid tumours at doses ranging from 0.2 to 838mg/m^2 infused over a 10-minute period. Pemetrexed has a steady-state volume of distribution of 9 l/m^2. *In vitro* studies indicate that pemetrexed is approximately 81% bound to plasma proteins. Binding was not notably affected by varying degrees of renal impairment. Pemetrexed undergoes limited hepatic metabolism. Pemetrexed is primarily eliminated in the urine, with 70% to 90% of the administered dose being recovered unchanged in urine within the first 24 hours following administration. Pemetrexed total systemic clearance is 91.8ml/min and the elimination half-life from plasma is 3.5 hours in patients with normal renal function (creatinine clearance of 90ml/min). Between patient variability in clearance is moderate at 19.3%. Pemetrexed total systemic exposure (AUC) and maximum plasma concentration increase proportionally with dose. The pharmacokinetics of pemetrexed are consistent over multiple treatment cycles.

The pharmacokinetic properties of pemetrexed are not influenced by concurrently administered cisplatin. Oral folic acid and intramuscular vitamin B_{12} supplementation do not affect the pharmacokinetics of pemetrexed.

5.3 Preclinical safety data

Administration of pemetrexed to pregnant mice resulted in decreased foetal viability, decreased foetal weight, incomplete ossification of some skeletal structures, and cleft palate.
Administration of pemetrexed to male mice resulted in reproductive toxicity characterised by reduced fertility rates and testicular atrophy. In a study conducted in beagle dog by intravenous bolus injection for 9 months, testicular findings (degeneration/necrosis of the seminiferous epithelium) have been observed. This suggests that pemetrexed may impair male fertility. Female fertility was not investigated.
Pemetrexed was not mutagenic in either the *in vitro* chromosome aberration test in Chinese hamster ovary cells, or the Ames test. Pemetrexed has been shown to be clastogenic in the *in vivo* micronucleus test in the mouse.
Studies to assess the carcinogenic potential of pemetrexed have not been conducted.

6. PHARMACEUTICAL PARTICULARS

6.1 List of excipients

Mannitol
Hydrochloric acid
Sodium hydroxide

6.2 Incompatibilities

Pemetrexed is physically incompatible with diluents containing calcium, including lactated Ringer's injection and Ringer's injection. In the absence of compatibility studies this medicinal product must not be mixed with other medicinal products.

6.3 Shelf life

Two years.
Reconstituted and infusion solutions: When prepared as directed, reconstituted and infusion solutions of Alimta contain no antimicrobial preservatives. Chemical and physical in-use stability of reconstituted and infusion solutions of pemetrexed were demonstrated for 24 hours at refrigerated temperature or 25°C. From a microbiological point of view, the product should be used immediately. If not used immediately, in-use storage times and conditions prior to use are the responsibility of the user and would normally not be longer than 24 hours at 2 to 8°C, unless reconstitution/dilution has taken place in controlled and validated aseptic conditions.

6.4 Special precautions for storage

Unopened vial: This medicinal product does not require any special storage conditions.
For storage conditions of the reconstituted medicinal product see section 6.3.

6.5 Nature and contents of container

Powder in Type I glass vial. Rubber stopper.
Pack of 1 vial.

6.6 Special precautions for disposal and other handling

1. Use aseptic technique during the reconstitution and further dilution of pemetrexed for intravenous infusion administration.
2. Calculate the dose and the number of Alimta vials needed. Each vial contains an excess of pemetrexed to facilitate delivery of label amount.
3. Reconstitute 500mg vials with 20ml of sodium chloride 9mg/ml (0.9%) solution for injection, without preservative, resulting in a solution containing 25mg/ml pemetrexed. Gently swirl each vial until the powder is completely dissolved. The resulting solution is clear and ranges in colour from colourless to yellow or green-yellow without adversely affecting product quality. The pH of the reconstituted solution is between 6.6 and 7.8. **Further dilution is required.**
4. The appropriate volume of reconstituted pemetrexed solution should be further diluted to 100ml with sodium chloride 9mg/ml (0.9%) solution for injection, without preservative, and administered as an intravenous infusion over 10 minutes.
5. Pemetrexed infusion solutions prepared as directed above are compatible with polyvinyl chloride and polyolefin lined administration sets and infusion bags.
6. Parenteral medicinal products should be inspected visually for particulate matter and discolouration prior to administration. If particulate matter is observed, do not administer.
7. Pemetrexed solutions are for single use only. Any unused product or waste material should be disposed of in accordance with local requirements.

Preparation and administration precautions: As with other potentially toxic anti-cancer agents, care should be exercised in the handling and preparation of pemetrexed infusion solutions. The use of gloves is recommended. If a pemetrexed solution contacts the skin, wash the skin immediately and thoroughly with soap and water. If pemetrexed solutions contact the mucous membranes, flush thoroughly with water. Pemetrexed is not a vesicant. There is not a specific antidote for extravasation of pemetrexed. There have been few reported cases of pemetrexed extravasation, which were not assessed as serious by the investigator. Extravasation should be managed by local standard practice as with other non-vesicants.

7. MARKETING AUTHORISATION HOLDER

Eli Lilly Nederland BV, Grootslag 1-5, NL-3991 RA Houten, The Netherlands.

8. MARKETING AUTHORISATION NUMBER(S)

EU/1/04/290/001

9. DATE OF FIRST AUTHORISATION/RENEWAL OF THE AUTHORISATION

Date of first authorisation: 20 September 2004
Date of renewal of authorisation: -

10. DATE OF REVISION OF THE TEXT

14 March 2007

LEGAL CATEGORY

POM

*ALIMTA (pemetrexed) is a trademark of Eli Lilly and Company.

AT4M

http://emc.medicines.org.uk/emc/assets/c/html/displaydoc.asp?documentid=15513

SUPPORTING INFORMATION

Patient Information Leaflet:
Alimta 500mg powder for concentrate for solution for infusion

Alternative format PIL:
View X-PIL (new window)

D16

Eli Lilly and Company Limited

Dextra Court
Chapel Hill
Basingstoke
Hampshire RG21 5SY
Telephone +44 (0) 1256 315000
Fax +44 (0) 1256 315858

Customer Care Line: +44 (0) 1256 315999

14th September 1999

[redacted]

Dear [redacted]

LETTER OF AGREEMENT - TWO WAY WITHOUT ADVANCE

Eli Lilly and Company Limited (Lilly) appreciates having the opportunity to collaborate with you (hereinafter referred to as "You" or "Your" in this study entitled "A Phase II Trial of MTA Administered Intravenously every 21 Days in Patients with Malignant Pleural Mesothelioma" (protocol) H3E/MC/JMDR, which Protocol is incorporated herein by reference Study. This Agreement sets forth Your obligations as the Investigator and those of Lilly as the sponsor.

I. YOUR OBLIGATIONS

You agree to assume the following obligations in executing this Agreement:

A. Conduct of the Study

You agree to personally conduct or supervise the Study at Your facility or institution. You agree to comply with: all conditions specified in the Protocol and Protocol amendments and/or addenda; applicable requirements of the Declaration of Helsinki; applicable Good Clinical Practice Guidelines and/or other nationally established guidelines; the approval of Your Ethical Review Board (ERB); and all other applicable laws or standards. You shall ensure that all of Your associates, colleagues and employees involved in the conduct of the Study at Your facility or institution also understand and comply with these obligations. You shall obtain the written consent of the institution in which the Study is to be performed to Your conduct of the trial.

If You are not a licensed physician, You shall ensure that a licensed physician is an investigator or sub-investigator at Your site and will be responsible for patient care and other appropriate aspects of this Study.

You acknowledge that You have read and understand all information in the investigator's brochure provided to You by Lilly, including the potential risks and side effects of the Study drug.

You agree that no investigations, or procedures, other than those specified by the Protocol or required for normal routine medical care, will be performed on the Study patients without the prior agreement of Lilly and the approval of Your ERB.

You agree to inform the Study patient's primary care physican of the patient's participation in the Study.

You agree to ensure the accuracy, completeness, legibility and timeliness of the Study data reported in the case record forms (CRF) and in all required reports. Data reported on the CRF, that are derived from source documents, should be consistent with the source documents or discrepancies should be explained.

You agree not to pay fees to another physician for the referral of patients. You shall not allow a subject to be enrolled simultaneously in this Study and another clinical trial without Lilly's advance written permission.

You agree to only use an informed consent document which has been reviewed and approved by Lilly.

You agree that Lilly or Lilly-designated representatives and domestic or foreign regulatory agencies may inspect Your procedures, facilities and Study records (including portions of other pertinent records for all patients in the Study) and those procedures, facilities or Study records of any employee, contractor or agent or Study site that You use in conducting the Study. Information obtained from such inspections may be shared with Lilly and Lilly-designated representatives. In the event that Lilly or Lilly-designated representatives discover that there is a lack of compliance with this Agreement, the Protocol, Good Clinical Practice Guidelines, applicable government regulations or other regulatory requirements, Lilly is entitled to secure compliance or discontinue shipments of Study drug and end Your participation in the Study.

B. Study Drug Use and Record Retention

Drugs furnished for the Study will be used solely under the Protocol and may not be used for any other purposes. You shall follow Lilly's instructions related to disposition of clincial trial materials. You shall be responsible for compliance with all laws and regulations applicable to any destruction or disposition of clinical materials at Your site. All Study records must be retained for fifteen (15) years after completion or termination of the Study. After this time you will contact Lilly who may require storage for a longer period of time. In such case Lilly will pay a storage fee to defray the cost of continuing storage. After this time you will contact Lilly who may require storage for a longer period of time. In such case Lilly will pay a storage fee to defray the cost of continuing storage.

C. Study Review Meetings

You agree to meet with Lilly representatives for Study review meetings at times agreed with Lilly to discuss the progress of the Study, and the parties' respective levels of satisfaction with it. At these meetings, the parties shall negotiate in good faith any required variations to the conduct of the Study and consequent adjustments required in order to address any areas of dissatisfaction.

The parties shall agree the time, date and venue of these meetings and will prepare an agenda for discussion at the meeting. Attendees of the meeting shall include those representatives with authority to make decisions on behalf of the parties.

Minutes of the meeting shall be prepared and agreed by both parties as soon after the meeting as possible.

D. Confidentiality and Non-Use

All information which is not already in the public domain provided to You by Lilly or Lilly designated representatives or generated by You in connection with the Study will be kept in confidence and not used for any purpose not contemplated by this Agreement for at least ten (10) years after the termination or conclusion of the Study, except to the extent that Lilly gives You written permission or particular information is required by laws or regulations to be disclosed to the ERB, the patient or regulatory agencies. To the extent disclosure is requested by any other person or entity, You shall promptly notify Lilly and shall not disclose any information without Lilly's prior written consent or until Lilly has exhausted any legal actions it may take to prevent or limit the requested disclosure. You shall be responsible for ensuring that Your employees, contractors and agents are obligated to these same terms of confidentiality and non-use. The terms of confidentiality and non-use set forth herein shall supersede any prior terms of confidentiality and non-use agreed to by the parties in connection with this Study and/or the Study drug. The terms of this Agreement shall also be considered confidential information, but may be disclosed only to the extent required by law or necessary for approval of this Study at your institution.

E. Data and Publications

Data generated in connection with the Study shall be the sole property of Lilly and shall be subject to the obligations of confidentiality and non-use set forth in Section D above; provided, however, You will be free to publish and present the results of the Study subject to the following conditions: Lilly will be furnished with a copy of any proposed publication or presentation for review and comment sixty (60) days prior to such presentation or submission for publication. At the expiration of such sixty (60) day period, You may proceed with the presentation or submission for publication; provided, however, that in the event Lilly has notified You in writing that Lilly reasonably believes that prior to such publication or presentation it must take action to protect its intellectual property interests, such as the filing of a patent application claiming an invention or a trademark registration application, or taking action to protect its data package exclusivity interests, You shall either (1) delay such publication or presentation for an additional ninety (90) days or until the foregoing action(s) have been taken, whichever shall first occur, or (2) if You are unwilling to delay the publication, You will remove from the publication or presentation the information which Lilly has specified it reasonably believes would jeopardize its intellectual property interests. Under certain circumstances, a shorter review period may be granted in writing by Lilly. You will assist Lilly in obtaining reprints of Your publication(s) resulting from the Study.

F. Inventions

If during the course of the Study, You conceive or actually reduce to practice what You believe to be a new invention or use involving the Study drug(s), You will promptly notify Lilly. The new invention or use shall be the sole property of and all rights in it shall be assigned to Lilly.

G. Publicity

1) Solicitation of patients. Lilly and Your ERB must approve, in writing, the text of any communication soliciting patients for the Study before placement, including, but not limited to, newspaper and radio advertisements, direct mail pieces, Internet advertisements or communications, and newsletters. Such communications must comply with applicable laws, guidelines and codes of practice and, in particular, shall not name the Study drug(s), contain therapeutic claims or mention Lilly.

2) Press releases. Lilly must approve, in writing, press statements by You regarding the Study or the Study drug(s) before the statements are released.

3) Inquiries from media and financial analysts. During and after the Study You may receive inquiries from reporters or financial analysts. Lilly requests that You confer with Your designated Lilly Research Physician or the Medical Director at Eli Lilly and Company Limited, Dextra Court, Chapel Hill, Basingstoke, RG21 5SY (01256) 315000, before responding to such inquiries.

4) Use of Name. You will not use Lilly's name or trademarks, tradenames or logos or the names of any Lilly employees in any advertising or sales promotional material or in any publication without the prior written permission of Lilly. Lilly shall not use Your name or the names of any of Your employees in any advertising or sales promotional material or in any publication without Your prior written permission.

H. Debarment Certification (Generic Drug Enforcement Act of 1992)

You agree to submit to Lilly upon completion or termination of the Study a certification that You have not been debarred by the United States Food and Drug Administration (FDA) and that You did not use, and will not use in any capacity in connection with the Study, any individual or person debarred by the FDA under the provisions of the United States Generic Drug Enforcement Act of 1992. If any person involved with this Study becomes debarred or is the subject of a debarment proceeding at any time during this Study, You will notify Lilly immediately.

I. CRO Involvement

Lilly shall be entitled to appoint a Contract Research Organisation (CRO) to perform all or any of the obligations owed by Lilly as Sponsor of the Study under prevailing guidelines relating to Good Clinical Practice. You will be notified of any such appointment or change of appointment and undertake to cooperate with any such CRO appointed on all Study related matters delegated to it by Lilly and to accept instructions from such CRO in relation to such matters as if the same were given by Lilly pursuant to this Agreement.

J. Equipment

If Lilly or a Lilly-designated representative is providing You with equipment for use in this Study (Equipment), You agree that Lilly shall not own or insure the Equipment, or be responsible for maintenance or any risk of loss in connection with the Equipment, during the term of the Study. You agree that the Equipment shall remain in the same condition during the Study, ordinary wear and tear excepted and that You will follow Lilly's instructions for disposition of the Equipment at the completion or termination of the Study.

II. LILLY SUPPORT

Lilly will provide You with Study drug(s). In addition, Lilly will provide financial support for the Study as follows:

A. Payee

If You are an independent investigator (i.e., You do not receive remuneration from the institution in which the Study will be performed, and payment to You under the terms of this Agreement will not violate any policy or agreement that You have with a third party with which You are affiliated), please acknowledge this fact by signing Your name and inserting the date below.

I am an independent investigator:

Signature [redacted] Date [redacted]

Unless You acknowledge that You are an independent investigator (see above), payments will be made to Your institution. If another payee is requested, a letter must be provided from a responsible official of Your institution which: (1) authorises payment to a payee other than Your institution, (2) states that such payments can be made in concert with the rules and policies of Your institution; and (3) certifies that such payment will not violate applicable laws or regulations.

Payment will be made to:

See exhibit A (attached)

(Identification Number for Tax Purposes)

B. Payment Schedule

The Budget attached hereto as Exhibit A (Budget) indicates that a maximum amount of [redacted] will be paid to You, Your institution or Your nominated payee in connection with the Study. For those amounts designated for patient services, You, Your institution or Your nominated payee will receive payment only for the actual number of visits and procedures performed in accordance with agreed upon procedure fees outlined in the Budget; such compensation is limited to payment for the number of patients designated in the Budget who are enrolled in the Study by , unless Lilly has given You written approval to enroll additional patients or extend the enrolment period.

When recruitment into a study is on a competitive basis there can be no guarantee that any individual site will have the full recruitment period in which to recruit patients or have a specific number of patients allocated.

To be eligible for payment, the procedures must be performed in full compliance with the Protocol and this Agreement, and the data submitted must be complete and correct. For data to be complete and correct, each patient must have signed an ERB-approved consent document, and all procedures designated in the Protocol must be carried out on a "best efforts" basis; omissions must be satisfactorily explained. It is expected that for all items required under the Protocol for which Lilly has agreed to provide compensation, Lilly will be the sole source of compensation.

Payments, if due, will be made based on the Budget and the data received, at Quarterly intervals; provided, however, that (1) other than the final payment, Lilly shall not issue any payment for a total amount less than Two Hundred Pounds (£200.00); (2) the final payment will be made when all patients have completed the Study and all available data and case report forms have been received and accepted by Lilly; and (3) matters in dispute shall be payable upon mutual resolution of such dispute. In the event the amount due in any given period is less than Two Hundred Pounds (£200.00), such amount shall carry-over without payment to the next payment period.

When Your data are reviewed by an on-site scheduled visit of a Lilly-designated representative, You will have all reasonably available data obtained through the preceding day complete and ready for evaluation. Lilly reserves the right to refuse payment for data not received by Lilly within ten (10) days after the representative's review.

In addition, if Lilly requests Your attendance at a Study start-up meeting or other meeting necessary to provide You information regarding the Study or Study Drug, Lilly shall reimburse You or Your institution for reasonable and necessary travel and lodging expenses that You incur to attend such meeting(s) and that have been specifically approved in advance by Lilly. Lilly shall make such reimbursements within thirty (30) days of receiving acceptable detailed documentation of such expenses, provided that Lilly receives such documentation within sixty (60) days of the date that the expenses were incurred.

C. Subject Injury Reimbursement

Lilly agrees to reimburse You for the following additional costs:

(1) All reasonable and customary costs incurred by You and associated with the diagnosis of an adverse event involving the Study drug or a Protocol procedure; and

(2) All reasonable and customary costs incurred for treatment of the subject if Lilly determines after consulting with You that the adverse event was reasonably related to administration of the Study drug or a Protocol procedure;

provided, however, that:

(a) such costs are not covered by the subject's medical or hospital insurance or other governmental programme providing such coverage;

(b) the adverse event is not attributable to Your or any agents', contractors' or employees' negligence or misconduct;

(c) the adverse event is not attributable to any underlying illness, whether previously diagnosed or not;

(d) the Study drug or Protocol procedure was administered in accordance with the Protocol; and

(e) the subject would not have undergone the protocol procedure which caused the adverse event but for the inclusion of the subject in the Study.

Lilly shall have the option of paying the additional costs directly to the provider of the service or to You.

D. Limit of Patient Entry or Enrolment and Study Termination

Lilly reserves the right to limit entry or enrolment of additional patients at any time. This may occur in a competitive-enrolment Study because sufficient patients have been entered by other investigators to complete the needs of the Study. Lilly also reserves the right to terminate Your or any patient's participation in the Study or the Study itself at any time for any reason. In addition if there has been no recruitment into the study within 6 months of start-up your site will be closed and this Agreement shall be terminated. In the event Your participation in the Study or the Study itself is terminated, You agree to return, retain, or dispose of all Study drug(s) in accordance with instructions to be provided by Lilly and regulatory requirements.

In the event of termination, payments will be made for all work that has been performed up to the date of termination and shall be limited to reasonable non-cancelable costs which were incurred by You in connection with the Study as required under the Protocol and contemplated in the Budget. If any payments exceed the amount owed for work performed under the Protocol, You agree to return the excess balance to Lilly.

III. INDEMNIFICATION

In consideration of the performance by You and Your staff, officers, agents and employees (Indemnitees) of the work described in the Protocol and submission to Lilly of a complete report of the results of the investigation, Lilly agrees to indemnify, defend and hold harmless the Indemnitees from and against loss, damage, cost and expense of claims and suits (including reasonable legal costs and expenses) resulting from an injury to a patient seeking damages alleged to have been directly caused or contributed to by any substance or procedure administered in accordance with the Protocol, including the cost and expense of handling such claims and defending such suits; provided, however, (1) that Indemnitees have adhered to and complied with all applicable laws and regulations (including, without limitation, obtaining informed consents and ERB approvals), the specifications of the Protocol and all recommendations furnished by Lilly for the use and administration of any drug or device described in the Protocol; (2) that Lilly is

promptly notified of any such claim or suit; (3) that the Indemnitees cooperate fully in the investigation and defense of any such claim or suit; (4) that Lilly retains the right to defend the lawsuit in any manner it deems appropriate, including the right to retain legal counsel of its choice; and (5) that Lilly shall have the sole right to settle the claim, provided, however, that Lilly shall not admit fault on Your behalf without Your advance written permission. In addition, Lilly's obligation of indemnification shall not extend to any loss, damage or expense arising from the negligence, willful malfeasance or malpractice by the Indemnitees, it being understood that the administration of any substance in accordance with the Protocol shall not constitute negligence or malpractice for purposes of this Agreement.

IV. SURVIVORSHIP CLAUSE

The obligations under Section I and Section III shall survive the expiration, termination, or cancellation of this Agreement.

V. INDEPENDENT CONTRACTOR

In conducting the Study, You will be acting as an independent contractor, and not as an agent, partner, or employee of Lilly. You will not have any authority to make agreements with third parties that are binding on Lilly.

This Agreement represents the entire understanding between the parties, and supersedes all other agreements, express or implied, between the parties concerning the subject matter hereof. This Agreement shall be governed by and construed in accordance with the law of Scotland and the parties hereby submit to the exclusive jurisdiction of the Court of Session in Scotland.

If the foregoing is acceptable to You, please sign the enclosed Agreements and return one original to [redacted] in the enclosed envelope. If You have any questions, please call [redacted]

Yours sincerely,

Eli Lilly and Company Limited.

[redacted]

Investigator

AGREED AND ACCEPTED

14/9/99
Date

14/5/99
Date

17.9.99.
Date

EXHIBIT A

Budget for Study H3E-MC-JMDR

REDACTED

D15

Lilly

Eli Lilly and Company Limited
Dextra Court
Chapel Hill
Basingstoke
Hampshire RG21 5SY
Telephone +44 (0) 1256 315000
Fax +44 (0) 1256 315858

Customer Care Line: +44 (0) 1256 315999

21 April 1999

[redacted]

Dear [redacted]

LETTER OF AGREEMENT

Eli Lilly and Company Limited ("Lilly") appreciates having the opportunity to collaborate with [redacted] ("Investigator") and [redacted] in the Study entitled "A Single-blind Randomized Phase 3 Trial of MTA plus Cisplatin versus Cisplatin in Patients with Malignant Pleural Mesothelioma" (protocol H3E/MC/JMCH, which Protocol is incorporated herein by reference "Study"). This Agreement sets forth the joint and several obligations of the Investigator and University (hereinafter referred to together as "You" or "Your") and the obligations of Lilly as the sponsor.

I. YOUR OBLIGATIONS

You agree to assume the following obligations in executing this Agreement:

A. Conduct of the Study

You agree to personally conduct or supervise the Study at Your facility or institution. You and Your colleagues agree to comply with: all conditions specified in the Protocol and Protocol amendments, including the statements required by Lilly in Your informed consent document; applicable requirements the Declaration of Helsinki (Somerset West, South Africa, 1996); applicable Good Clinical Practice Guidelines and/or other nationally established guidelines; the approval of Your Ethical Review Board ("ERB"); and all other applicable national, state, and local laws or standards. You shall ensure that all of Your associates, colleagues and employees involved in the conduct of the Study at Your facility or institution also understand these obligations.

You acknowledge that You have read and understand all information in the investigator's brochure provided to You by Lilly, including the potential risks and side effects of the Study drug.

You agree not to pay fees to another physician for the referral of patients.

You agree to only use an informed consent document which has been reviewed and approved by Lilly.

You agree that Lilly or Lilly-designated representatives may inspect Your procedures, facilities and Study records (including portions of other pertinent records for all patients in the Study) and those procedures, facilities or Study records of any contractor or agent that You use in conducting the Study. Information obtained from such inspections may be shared with Lilly and Lilly-designated representatives. In the event that Lilly or Lilly-designated representatives discover that there is a lack of compliance with this Agreement, the Protocol, Good Clinical Practice Guidelines, applicable government regulations or other regulatory requirements, Lilly is entitled to secure compliance or discontinue shipments of Study drug and end Your participation in the Study.

B. Study Drug Use and Record Retention

Drugs furnished for the Study will be used solely under the Protocol and may not be used for any other purposes. All Study records must be retained for fifteen (15) years after completion or termination of the Study.

C. Confidentiality and Non-Use

All information provided to You by Lilly or generated by You in connection with the Study will be kept in confidence and not used for any purpose not contemplated by this Agreement for at least ten (10) years after the termination or conclusion of the Study, unless Lilly gives You written permission or particular information is required by laws or regulations to be disclosed to the ERB, the patient, or regulatory agencies. To the extent disclosure is requested by any other person or entity, You shall promptly notify Lilly and shall not disclose any information without Lilly's prior written consent or until Lilly has exhausted any legal actions it may take to prevent or limit the requested disclosure. You shall be responsible for ensuring that Your employees and agents are obligated to these same terms of confidentiality and non-use. The terms of confidentiality and non-use set forth herein shall supersede any prior terms of confidentiality and non-use agreed to by the parties in connection with this Study and/or the Study Protocol. You shall not disclose any information related to this Study or the Protocol to any third party for the purpose of making such information (or a summary) available in any publication or electronic clinical trial databases. Consistent with this requirement, You agree to confer with Lilly Legal in the United States if You are requested to provide any such information for that purpose. Lilly Legal will negotiate the terms, at Lilly's sole discretion, under which such information shall be released, if at all.

D. Data and Publications

Data emanating from the Study shall be the sole property of Lilly and shall be subject to the obligations of confidentiality and non-use set forth in Section C above; provided, however, You will be free to publish and present the results of the Study subject to the following conditions: Lilly will be furnished with a copy of any proposed publication or presentation for review and comment sixty (60) days prior to such presentation or submission for publication. At the expiration of such sixty (60) day period, You may proceed with the presentation or submission for publication; provided, however, that in the event Lilly has notified You in writing that Lilly reasonably believes that prior to such publication or presentation it must take action to protect its intellectual property interests, such as the filing of a patent application claiming an invention or a trademark registration application, or taking action to protect its data package exclusivity interests, You shall either (1) delay such publication or presentation for an additional ninety (90) days or until the foregoing action(s) have been taken, whichever shall first occur, or (2) if You are unwilling to delay the publication, You will remove from the publication or presentation the information which Lilly has specified it reasonably believes would jeopardize its intellectual property interests. Under certain circumstances, a shorter review period may be granted in writing by Lilly. You will assist Lilly in obtaining reprints of Your publication(s) resulting from the Study.

E. Work Product

All reports, brochures, handouts, documents or other work product ("Work Product") developed or compiled by You in connection with performing services pursuant to this Agreement shall be the property of Lilly and shall be delivered to Lilly upon request or upon termination of this Agreement. The Work Product shall constitute a work for hire for Lilly and, therefore, Lilly shall own all copyrights arising from Your services under this Agreement; however, Work Product shall not include any publication reviewed and approved by Lilly in accordance with Section I(D) above. In the event any Work Product is not deemed to constitute a work for hire for Lilly, You hereby assign all rights, title and interest that You have or may acquire in the Work Product for Lilly. You agree to execute without further consideration all assignments or other documents that may be necessary or helpful to establish Lilly's ownership of the Work Product. All Work Product shall be original creations for Lilly and shall not infringe any patent, copyright or other proprietary right of a third party. If requested, any or all Work Product owned by Lilly shall be transferred to Lilly in a form and manner to be agreed upon by You and Lilly. The information provided to You by Lilly in order for You to carry out the services under this Agreement, including reports, brochures, documents, handouts, ideas, concepts, etc., and the Work Product shall at all times remain solely the property of Lilly and shall not be utilized in any manner beyond what is called for in this Agreement. Additionally, You shall not issue, release or disclose any information concerning the services provided under this Agreement to any person without prior review and approval by Lilly.

A. Inventions

If during the course of the Study, You conceive or actually reduce to practice what You believe to be a new invention or use involving the Study drug(s), You will promptly notify Lilly. The new invention or use shall be the sole property of and shall be assigned to Lilly.

G. Publicity

1) Solicitation of patients. Lilly and Your ERB must approve, in writing, the text of any communication soliciting patients for the Study before placement, including, but not limited to, newspaper and radio advertisements, direct mail pieces, and the Internet. Such communications shall not name the Study drug(s), contain therapeutic claims or mention Lilly.

2) Press releases. Lilly must approve, in writing, press statements by You regarding the Study or the Study drug(s) before the statements are released.

3) Inquiries from media and financial analysts. During and after the Study You may receive inquiries from reporters or financial analysts. Lilly requests that You confer with Your designated Lilly Research Physician or our Medical Director at Eli Lilly and Company Limited, Dextra Court, Chapel Hill, Basingstoke (01256) 315000, before responding to such inquiries.

4) Use of Name. You will not use Lilly's name or the names of any Lilly employees in any advertising or sales promotional material or in any publication without the prior written permission of Lilly. Lilly shall not use Your name or the names of any of Your employees in any advertising or sales promotional material or in any publication without Your prior written permission.

H. Debarment Certification (Generic Drug Enforcement Act of 1992)

You agree to submit to Lilly upon completion or termination of the Study a certification that You have not been debarred by the United Stated Food and Drug Administration ("FDA") and that You did not use, and will not use in any capacity in connection with the Study, any individual or person debarred by the FDA under the provisions of the United States Generic Drug Enforcement Act of 1992.

II. LILLY SUPPORT

Lilly will provide You with Study drug(s). In addition, Lilly will provide financial support for the Study as follows:

A. Payee

If You are an independent investigator (i.e., You do not receive remuneration from the institution in which the Study will be performed, and payment to You under the terms of this Agreement will not violate any policy or agreement that You have with a third party with which You are affiliated), please acknowledge this fact by signing Your name and inserting the date below.

I am an independent investigator:

_____ _____

Signature Date

Unless You acknowledge that You are an independent investigator (see above), payments will be made to Your institution. If another payee is requested, a letter must be provided from a responsible official of Your institution which: (1) authorizes payment to a payee other than Your institution, (2) states that such payments can be made in concert with the rules and policies of Your institution; and (3) certifies that such payment will not violate applicable federal, state, or local laws or regulations.

Payment will be made to:

[redacted]

_____

(Taxpayer I.D. or SS Number)

B. Payment Schedule

The Budget attached hereto as Exhibit A ("Budget") indicates that [redacted] will be paid to You in connection with the Study. For those amounts designated for patient services, You will receive payment only for the actual number of visits and procedures performed in accordance with agreed upon procedure fees outlined in the Budget; such compensation is limited to payment for the number of patients designated in the Budget who are enrolled in the Study by October 2000, unless Lilly has given You written approval to enroll additional patients or extend the enrolment period.

When recruitment into a study is on a competitive basis there can be no guarantee that any individual site will have the full recruitment period in which to recruit patients or have a specific number of patients allocated.

To be eligible for payment, the procedures must be performed in full compliance with the Protocol and this Agreement, and the data submitted must be complete and correct. For data to be complete and correct, each patient must have signed an ERB-approved consent document, and all procedures designated in the Protocol must be carried out on a "best efforts" basis; omissions must be satisfactorily explained. It is expected that for all items required under the Protocol for which Lilly has agreed to provide compensation, Lilly will be the sole source of compensation.

Payments, if due, will be made based on the Budget and the data received, at Quarterly intervals; provided, however, that (1) other than the final payment, Lilly shall not issue any payment for a total amount less than Two Hundred Pounds (£200.00); (2) the final payment will be made when all patients have completed the Study and all available data and case report forms have been received and accepted by Lilly; and (3) matters in dispute shall be payable upon mutual resolution of such dispute. In the event that the Advance credit in any given period exceeds the amount of a payment due, the excess credit shall carry-over and be applied against the subsequent payment, in addition to any otherwise applicable credit. In the event the amount due in any given period is less than Two Hundred Pounds (£200.00), such amount shall carry-over without payment to the next payment period.

When Your data are reviewed by an on-site scheduled visit of a Lilly-designated representative, You will have all reasonably available data obtained through the preceding day complete and ready for evaluation. Lilly reserves the right to refuse payment for data not received by Lilly within ten (10) days after the representative's review.

In addition, if Lilly requests Your attendance at a Study start-up meeting or other meeting necessary to provide You information regarding the Study or Study Drug, Lilly shall reimburse You for reasonable and necessary travel and lodging expenses that You incur to attend such meeting(s) and that have been specifically approved in advance by Lilly. Lilly shall make such reimbursements within thirty (30) days of receiving acceptable detailed documentation of such expenses, provided that Lilly receives such documentation within sixty (60) days of the date that the expenses were incurred.

C. Subject Injury Reimbursement

Lilly agrees to reimburse You for the following additional costs:

(1) All reasonable and customary costs incurred by You and associated with the diagnosis of an adverse event involving the Study drug or a Protocol procedure; and

(2) All reasonable and customary costs incurred for treatment of the subject if Lilly determines after consulting with You that the adverse event was reasonably related to administration of the Study drug or Protocol;

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(b) the adverse event is not attributable to Your or any agents' or employees' negligence or misconduct;

(c) the adverse event is not attributable to any underlying illness, whether previously diagnosed or not; and

(d) the Study drug or Protocol procedure was administered in accordance with the Protocol.

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Lilly reserves the right to limit entry or enrolment of additional patients at any time. This may occur in a competitive-enrolment Study because sufficient patients have been entered by other investigators to complete the needs of the Study. Lilly also reserves the right to terminate Your or any patient's participation in the Study or the Study itself at any time for any reason. In addition if there has been no recruitment into the study within 6 months of start-up your site will be closed. In the event Your participation in the Study or the Study itself is terminated, You agree to return, retain, or dispose of all Study drug(s) in accordance with instructions to be provided by Lilly and regulatory requirements.

In the event of termination, payments will be made for all work that has been performed up to the date of termination and shall be limited to reasonable non-cancelable costs which were incurred by You in connection with the Study as required under the Protocol and contemplated in the Budget. If the Advance or other payments exceed the amount owed for work performed under the Protocol, You agree to return the excess balance to Lilly.

III. INDEMNIFICATION

In consideration of the performance by You and Your staff, officers, agents and employees ("Indemnitees") of the work described in the Protocol and submission to Lilly of a complete report of the results of the investigation, Lilly agrees to indemnify, defend and hold harmless the Indemnitees from and against loss, damage, cost and expense of claims and suits (including reasonable attorneys' fees) resulting from an injury to a patient seeking damages alleged to have been directly caused or contributed to by any substance or procedure administered in accordance with the Protocol, including the cost and expense of handling such claims and defending such suits; provided, however, (1) that Indemnitees have adhered to and complied with all applicable federal, state and local regulations (including, without limitation, obtaining informed consents and ERB approvals), the specifications of the Protocol and all recommendations furnished by Lilly for the use and administration of any drug or device described in the Protocol; (2) that Lilly is promptly notified of any such claim or suit; (3) that the Indemnitees cooperate fully in the investigation and defense of any such claim or suit; (4) that Lilly retains the right to defend the lawsuit in any manner it deems appropriate, including the right to retain counsel of its choice; and (5) that Lilly shall have the sole right to settle the claim. In addition, Lilly's obligation of indemnification shall not extend to any loss, damage or expense arising from the negligence, willful malfeasance or malpractice by the Indemnitees, it being understood that the administration of any substance in accordance with the Protocol shall not constitute negligence or malpractice for purposes of this Agreement.

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In conducting the Study, You will be acting as an independent contractor, and not as an agent, partner, or employee of Lilly. You will not have any authority to make agreements with third parties that are binding on Lilly.

This Agreement represents the entire understanding between the parties, and supersedes all other agreements, express or implied, between the parties concerning the subject matter hereof. This Agreement shall be interpreted in accordance with the laws of England.

If the foregoing is acceptable to You, please sign the enclosed Agreements and return one original to [redacted] in the enclosed envelope. If You have any questions, please call [redacted]

Yours sincerely,

Eli Lilly and Company Limited .

AGREED AND ACCEPTED

[redacted]

28th April 1999
Date

[redacted]

6 MAY 99
Date

[redacted]
Investigator

5/5/99
Date

[redacted]
Print Name/Title

Agreed and Accepted [redacted]

[redacted]
Authorised Official

27/5/99
Date

[redacted]
Print Name/Title

EXHIBIT A

Budget for Study H3E-MC-JMCH

REDACTED

Arzneimittel-wirkungen

Lehrbuch der Pharmakologie und Toxikologie

Mit einführenden Kapiteln
in die Anatomie, Physiologie und Pathophysiologie

von

Dr. rer. nat. Dr. med. Ernst Mutschler

o. Professor für Pharmakologie
im Fachbereich Biochemie, Pharmazie und Lebensmittelchemie
der Johann-Wolfgang-Goethe-Universität Frankfurt/Main

5., völlig neubearbeitete und erweiterte Auflage

189 Abbildungen, 279 Formelbilder und 160 Tabellen

Wissenschaftliche Verlagsgesellschaft mbH Stuttgart
1986

10.3. Antimetaboliten

Antimetaboliten verdrängen *kompetitiv* natürliche Stoffwechselbausteine (Metaboliten) oder blockieren Enzyme und hemmen auf diese Weise den Stoffwechsel und das Zellwachstum. Ihre Wirkung ist weitgehend *unspezifisch*, d. h. der Stoffwechsel *aller* sich schnell teilender Zellen wird in gleicher Weise betroffen. Aus diesem Grund sind Antimetaboliten hochtoxisch, was ihre Anwendung weitgehend einschränkt.

10.3.1. Folsäureantagonisten

Durch geringfügige chemische Abwandlung der Folsäure wurden Folsäureantagonisten erhalten, die eine wesentlich höhere Affinität zur Dihydrofolsäure-Reduktase als Folsäure selbst besitzen und auf diese Weise die Übertragung von Einkohlenstoff-Fragmenten (s. S. 388) verhindern. Die Folge ist eine gestörte Nucleinsäuresynthese.
In die Therapie wurden *Aminopterin* und *Methotrexat* eingeführt, von denen Aminopterin bereits wieder aus dem Handel gezogen werden mußte.
Folinsäure (Citrovorum-Faktor, Leucovorin®), nicht aber Folsäure ist ein wirksames *Antidot.*

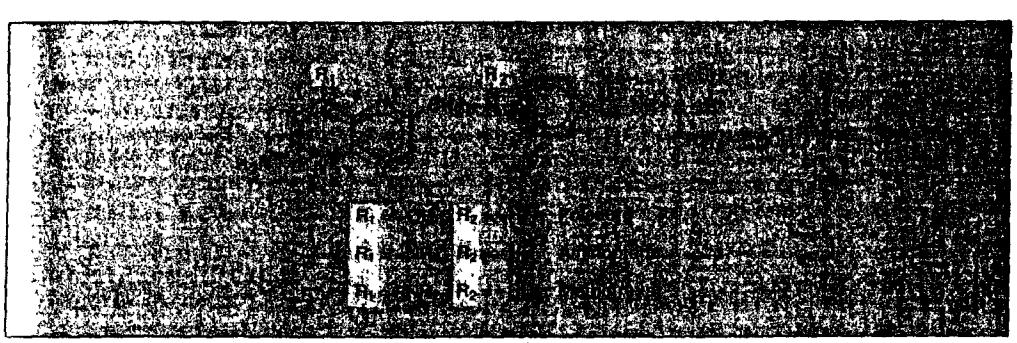
Methotrexat (Methotrexat Bristol, Methotrexat „Lederle", Methotrexat Rhone-Poulenc) wird vorwiegend bei akuten Leukämien, Chorionepitheliom und verschiedenen Karzinomen, ferner bei Autoimmunerkrankungen (s. S. 656) eingesetzt.

Die *Dosierung* hängt in hohem Maße vom Behandlungsschema ab. Bei den heute z. T. verwendeten Hochdosierungen (1–20 g) wird davon ausgegangen, daß zunächst die Tumorzellen und erst später andere Körperzellen durch Methotrexat beeinflußt werden und es dadurch möglich ist, durch rechtzeitige Gabe des Antidots Citrovorum-Faktor die Körperzellen vor der Zerstörung zu retten (sog. Citrovorum-Faktor-Rescue).
Die antineoplastische Wirkung solch exzessiver Methotrexat-Gaben beruht darauf, daß Methotrexat in hoher intrazellulärer Konzentration dann auch die für die Resistenzentwicklung verantwortliche niedrig affine Dihydrofolat-Reduktase zu hemmen vermag.

10.3.2. Antagonisten von Purin- und Pyrimidin-Basen

Zu den Purin-Analogen gehören *Mercaptopurin* und *Tioguanin*, zu den Pyrimidin-Analogen *Fluorouracil* und *Cytarabin.*

Mercaptopurin (6-Mercaptopurin, Puri-Nethol®) kann entweder als Adenin- oder Hypoxanthin-Analogon (Ersatz der NH_2- des Adenins bzw. der OH-Gruppe des Hypoxanthins durch eine SH-Gruppe) aufgefaßt werden. Es wirkt als kompetitiver Hemmstoff bei der Purinbiosynthese. Die intrazelluläre Wirkform ist das *6-Mercaptopurin-ribonucleotid.* Durch die Hemmung verschiedener Enzyme, u. a. der Adenylosuccinat-Synthetase und der Phosphoribosylpyrophosphatamido-Transferase, werden die



Pharmakologie und Toxikologie

Lehrbuch für Studierende der Medizin, Pharmazie und Naturwissenschaften

Herausgegeben von

C.-J. Estler, Erlangen

Mit Beiträgen von

B. Ahlemeyer, Marburg
H. P. T. Ammon, Tübingen
K. von Bergmann, Bonn
K. W. Bock, Tübingen
K. Brune, Erlangen
C.-J. Estler, Erlangen
K. J. Freundt, Mannheim
H.-H. Frey, Neustadt i. H.
J. Greven, Aachen
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und 305 Tabellen

Schattauer Stuttgart New York

Prof. Dr. med. C.-J. Estler
Institut für Experimentelle und Klinische
Pharmakologie und Toxikologie der
Friedrich-Alexander-Universität Erlangen-Nürnberg,
Fahrstraße 17, 91054 Erlangen

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Tab. 21-1. Wichtige Indikationen und Nebenwirkungen von Zytostatika

Zytostatikum	Hauptindikationen	Generelle Nebenwirkungen	Spezielle Nebenwirkungen
a) N-Lostderivate	Lymphosarkome, lymphatische u. myeloische Leukämie, Hodgkin-Krankheit, solide Tumoren verschiedener Organe, bes. Ovarial-, Mamma- u. Bronchialkarzinom	Frühreaktionen: ● Übelkeit, Erbrechen ● Fieber ● Frösteln oder Schwitzen ● Abgeschlagenheit ● allgemeines Unwohlsein	lokale Unverträglichkeit Schädigung der ableitenden Harnwege (alle) psychische Störungen (Ifosfamid), Herzmuskelschwächen (Cyclophosphamid), Gynäkomastie (Estramustin)
b) Ethylenimine	ähnlich wie N-Lostderivate, Retinoblastom	Störung der Hämatopoese: ● Anämie ● Granulozytopenie ● Lymphopenie ● Thrombopenie	psychische Störungen, Erythrodermie
c) Alkylsulfonate Busulfan	Leukämie		Leberschäden
Treosulfan	Ovarialtumoren		
d) Nitrosoharnstoffderivate	ähnlich wie N-Lostderivate, Hirntumoren (Lomustin), Melanome, maligne Lymphome (Carmustin), Prostatakarzinom (Estramustin)	Immunsuppression Störung der Regeneration des Intestinalepitheis: ● aregeneratorische Enteropathie ● Stomatitis ● Enteritis ● Proktitis ● Malabsorption	Funktionsstörungen des ZNS, der Niere u. der Leber, Lungenfibrose
e) Cisplatin Carboplatin	solide Tumoren verschiedener Organe		irreversible Nierenschäden, Herz-Kreislauf- u. Elektrolytstoffwechselstörungen, periphere Neuropathien, Hörverlust, Sehstörungen
f) Dacarbazin	Melanome, Sarkome, Lymphome	Störung des Haarwachstums	Lebervenenverschluß, »grippeähnliche« Beschwerden, lokale Unverträglichkeit, Venenreizung
g) Procarbazin	Lymphome	Störung der Spermatogenese und Follikelreifung (Ovulation)	psychische Störungen, MAO-Hemmung, Alkoholunverträglichkeit, irreversible Infertilität
h) Mitomycin	solide Tumoren verschiedener Organe	Störung des Embryonal- und Fetalwachstums	Leber-, Nieren-, Lungenschäden
i) Dactinomycin	Rhabdomyosarkom, Wilms-Tumor, Chorionepitheliom u. a.	Hyperurikämie	lokale Gewebsschäden
j) Anthracycline Daunorubicin Aclarubicin Idarubicin	Leukämie		Kardiomyopathie: Arrythmie, Herzversagen, glykosidrefraktäre Myokardinsuffizienz (Letalität 50%!)
Doxorubicin Epirubicin	Leukämie, maligne Lymphome, solide Tumoren verschiedener Organe		
k) Amsacrin	lymphatische u. myeloische Leukämie		Funktionsstörungen von ZNS, Herz u. Leber, Augenschädigungen
l) Mitoxantron	Leukämie, maligne Lymphome, Mammakarzinom		Kardiomyopathie
m) Methotrexat	lymphatische u. myeloische Leukämie, Chorionepitheliom, solide Tumoren verschiedener Organe, Mycosis fungoides, Psoriasis, Non-Hodgkin-Lymphome		Leber- u. Nierenfunktionsstörungen, Lungenfunktionsstörungen, Osteoporose

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VITAMIN B_{12} AS A POSSIBLE ADJUNCT IN PREVENTION OF METHOTREXATE HEPATOTOXICITY

Anthony J. Barak, Harriet C. Beckenhauer and Dean J. Tuma

Liver Study Unit, Veterans Administration Hospital
and the Department of Internal Medicine and
Biochemistry, University of Nebraska Medical Center
Omaha, NE. 68105 U.S.A.

Abstract

It has been established by several investigators that hepatic betaine levels reflect whether or not a lipotrope deficiency state exists in the experimental animal.

Since methotrexate through its antagonism of folate metabolism in the organism may in turn interfere with one carbon metabolism, the possibility exists that methotrexate hepatotoxicity may be due to a drug-imposed lipotrope deficiency.

Several studies are reviewed in which the effects of methotrexate administration on levels of hepatic betaine were measured in rats. It was observed that animals receiving dietary vitamin B_{12} were protected against a methotrexate-induced lowering of hepatic betaine. The livers of those animals on a vitamin B_{12} deficient diet, however, showed a marked reduction in this important methylating agent when administered methotrexate. Since betaine is a reflection of lipotrope deficiency, the use of vitamin B_{12} as a means of protecting against methotrexate hepatotoxicity is considered.

It is well known that methotrexate (MTX), a folate antagonist used in cancer chemotherapy and psoriasis, inhibits the enzyme dihydrofolate reductase. This inhibition reduces the amount of tetrahydrofolate coenzymes (1) available for purine synthesis, and in turn, reduces nucleic acid formation in the body hence restricting rapidly growing tissue. A common practice in treating neoplastic disease has been to administer citrovorum-factor along with the MTX in order to "rescue" the normal cells from the deleterious effects of inhibited folate metabolism. Since tumor cells are more vulnerable than normal cells to the lack of nucleic acid synthesis, the citrovorum factor furnishes needed folate coenzymes and helps rescue normal cells in the wake of the methotrexate effects.

If the tetrahydrofolate coenzyme pool of the body is depleted by the action of MTX, it is entirely feasible that this may impair certain vital methyl transfer reactions in the liver. For example, this may inhibit the N^5-methyltetrahydrofolate-homocysteine methyltransferase involved in the

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formation of lipotropic substances such as methionine, S-adenosylmethionine (SAM) and choline. Since these substances are the major lipotropic compounds in the body, a serious deficiency could lead to fatty metamorphosis in the liver and the liver damage seen with the use of MTX.

Several workers have attempted to approach this question by conducting "protective" experiments with choline and other lipotropes when administering MTX to humans and animals. Weinstein and Frei (2) have suggested that concomitant administration of choline with methotrexate may prevent MTX-related hepatotoxicity in humans. Freeman-Narrod et al. (3) administered MTX alone and in combination with varying amounts of choline to rats. The rats receiving the combined treatment showed significantly lower levels of triglyceride in their livers than did those given MTX alone and the protective effect of the choline on the liver appeared to be dose related.

A past study in this laboratory (4), using the rat as the experimental model, has lent support to the findings of the above authors. This work tested the effect of MTX on hepatic fatty infiltration (a measure of liver injury) in rats fed a lipotrope-deficient diet and the effect of MTX on similarly fed animals when the diet was individually supplemented with the lipotropes methionine and choline. It was found that triglyceride and cholesterol esters were elevated in the rats fed the basal diet and that this accumulation was potentiated in animals receiving MTX. In animals whose diets were supplemented with choline or methionine, however, the drug did not affect the hepatic levels of triglyceride and cholesterol esters. These results indicated that MTX may interfere with the formation of choline and methionine but does not interfere with the lipotropic action of preformed choline and methionine. Since choline and methionine are products of one-carbon metabolism beyond the involvment of MTX with folate, these results support the case for supplementation with lipotropic agents during MTX administration.

Both choline and methionine are lipotropic substances by virtue of their contribution to phosphatidylcholine synthesis which is involved in proper cellular membrane function and structure (5). Methionine makes its contribution in this respect by its conversion to SAM which plays a vital role in sequential phospholipid methylation to produce essential phosphatidylcholine. The contribution of choline takes place in two different ways. One, through conversion to phosphatidylcholine in the Kennedy pathway (6) and secondly, via the oxidative pathway for choline which forms betaine. Betaine is then utilized by betaine-homocysteine methyltransferase to methylate homocysteine in an alternate pathway to form methionine and hence SAM.

According to Finkelstein and Martin (7), the reaction mediated by N^5-methyltetrahydrofolate-homocysteine methyltransferase and the reaction utilizing betaine through the medium of betaine-homocysteine methyltransferase are of equal importance in methionine synthesis and in promoting methylation in the liver. Once thought to fulfill a very minor function in the organism, betaine is now felt to play a very active and important role as a methylating substance and therapeutic agent (8,9). Some workers feel that the hepatic betaine level is a good index of a choline deficiency in the liver. Wong and Thompson (10), Barak and Tuma (11), and Martin and Finkelstein (12) have all shown a marked reduction in hepatic betaine levels in animals fed diets deficient in choline.

Since it was felt that hepatic betaine levels reflected the choline deficiency state of the animal, several studies (13-15) were conducted in this laboratory to determine the effect of MTX administration on hepatic betaine

levels in the rat. In this work, rats were divided into pairs and pair-fed with each of three different kinds of widely used control diets. These were the control diet of Iseri et al. (16), the control diet of French (17) and Purina rat chow, respectively. The experimental animals in each pair were injected daily and intraperitoneally with 0.1mg MTX/kgm body weight. Control animals were injected daily with saline. Rats fed the Iseri control diet showed no changes in hepatic betaine when treated with MTX for 10 days. Rats fed the Purina rat chow showed no changes in liver betaine in 30 days on the drug. However, rats fed the French control diet demonstrated a highly significant lowering of betaine in their livers with MTX treatment for 10 days.

The results summarized above posed the strong question of why MTX should produce a choline (betaine) deficiency state (10-12) when rats were fed the French control diet but not when fed the Iseri control diet or the Purina rat chow in the times described. On close study, it was determined that the major difference between the three diets used, other than the fat, was the vitamin B_{12} content. The French control diet, either as originally described or as supplied commercially, is devoid of vitamin B_{12}.

To determine whether the absence of dietary vitamin B_{12} was responsible for the lowering of hepatic betaine in the livers of rats receiving MTX, a further study (18) was conducted in this laboratory. Rats were divided into pairs. One group of paired rats was fed on the French choline deficient diet to which had been added choline at the level of 0.4 g per 100g of diet and vitamin B_{12} at the level of 2.2 µg per 100g of diet. A second group of paired rats was fed the same diet from which the B_{12} had been eliminated. Control animals of each pair in both groups were pair-fed to the consumption of the experimental animals in each group which were treated with MTX for 10 days. On sacrifice, it was shown that hepatic betaine was not reduced by the MTX in animals receiving the dietary B_{12}, however, the hepatic betaine was markedly lowered in those animals receiving MTX with no B_{12}.

At present the reason for the sparing effect of vitamin B_{12} for hepatic betaine is not clear. One would expect that hepatic betaine would be depleted by MTX even when dietary B_{12} is furnished. This should occur through the inhibition of the folate contribution to methionine biosynthesis with the pool size of betaine becoming lowered through the action of betaine-homocysteine methyltransferase as a compensatory mechanism to maintain essential methionine. This did not happen, however, and only with the lack of B_{12}, a coenzyme for N-methyltetrahydrofolate-homocysteine methyltransferase, was the betaine depleted.

It is feasible that the dietary presence of B_{12} may maintain or stimulate the action of N^5-methyltetrahydrofolate-homocysteine methyltransferase in the face of the MTX to preserve hepatic betaine or act in some unknown manner to increase betaine production through choline oxidase. Whatever the function of the B_{12}, the fact that this vitamin prevented a condition of hypomethylation from occurring in the experimental animal may prove to have practical application in the prevention of liver injury during MTX chemotherapy in humans.

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INFLUENCE OF METHYLCOBALAMIN ON THE ANTINEOPLASTIC ACTIVITY OF METHOTREXATE

F. G. Arsenyan, N. V. Myasishcheva, Z. P. Sol'ina, M. O. Raushenbakh, I. P. Rudakova, E. G. Chauser, and A. M. Yurkevich

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One of the possible ways of increasing the selectivity of the action of chemotherapeutic substances on tumor cells is the combined use of preparations, taking the peculiarities of the mechanism of their action into account. A new trend in this field is the use of cobalamin derivatives in combination with definite antineoplastic preparations.

The special significance of methylcobalamin was first noted in the case of impaired cobalamin metabolism in leukemia patients. An analysis of the functional activity of cobalamin coenzymes in the organism, in comparison with the effectiveness of combined cytostatic therapy, has shown that the clinical course of the process in acute leukemia with an increased content of hydroxy- and methylcobalamins in the blood is less favorable [1]. The results obtained were evidence of the important role of methylcobalamin in metabolic processes as a coenzyme of methionine synthetase (EC 2.1.1.13)—a key link in the control of the synchronized action of cobalamins in compounds of folic acid in processes of cell proliferation [1-2].

A study of the morphofunctional state of the hemopoietic system of animals under conditions of intensive cobalamin metabolism in the organism confirmed the fact that at a high concentration of cobalamin coenzymes, the rate of proliferation of cells of the hemopoietic tissue increases. In the spleens of healthy mice, in the case of prolonged administration of methylcobalamin, hyperplasia of the lymphoid elements, an increase in the number of DNA-synthesizing cells, and an increase in their mitotic index were noted. The stability of the periods of the mitotic cycle of spleen lymphocytes in the presence of an increase in the size of the proliferative

TABLE 1. Stimulating Effects of Methylcobalamin on the Growth of Transplantable Tumors of Mice

Tumor	Line of mice	Dose of prep., mg/kg	Increase in tumor volume after administration of methylcobalamin, % of control		
			7-8th day*	10-14th day*	21st day*
Ca-755	SDP,	10	160	+68	+10†
	C_{57}BL	10	+75	+40†	+77
	F,	500	+45	+10†	+30
	F,	500	+77	+29†	+52
AKATOL	BALB.c	10	+126	+37	−33
P2h14-8	CBA	10	+47	0	0
Sarcoma 37	SHK	500	+87	0	0

*Period after transplantation of tumor.
†$P > 0.05$, in all remaining cases $P < 0.05$.
Note. Here and in Table 2, the preparation was administered on the second and sixth days after transplantation of the tumor. A "plus" sign denotes stimulation of tumor growth.

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TABLE 2. Results of Combined Action of Methylcobalamin and Methotrexate on the Growth of Ca-756 (BDF_1)

Preparations	Dose of preparation	Inhibition of tumor growth* after course of administration of preparations, % of control		Increase in lifetime of animals, %
		1st-2nd day†	7-8th day†	
Methotrexate	10 mg/kg	94	51	19‡
Methylcobalamin	10 μg/kg	+180	+65	0
Methylcobalamin + methotrexate	10 mg/kg (simultaneously) 10 μg/kg	94	78	60
Methylcobalamin + methotrexate	10 μg/kg (methotrexate was administrated 6 h after methylcobalamin)	+36	+62	21‡

*Average results of five series of experiments.
†Period after transplantation of tumor.
‡$P > 0.05$; in all remaining cases $P < 0.05$. In the case of combined influence, the results obtained were evaluated relative to methotrexate.

pool made it possible to conclude that the intensified proliferation of hemopoietic cells under these conditions is due to an increase in the number of cells entering the mitotic cycle [3-5]. Further experimental investigations revealed the active role of methylcobalamin not only in processes of proliferation of cells of the hemopoietic tissue. An analogous influence on proliferative activity (an increase in the fraction of cells labeled with [^{3}H]thymidine and an increase in the mitotic index) has also been detected in various periods of culturing of embryonic human fibroblasts in media with a high methylcobalamin concentration [6-7].

In view of the fact that normal cells of adult animals, embryonic and tumor cells differ in their ability to respond to the inducing influence of cobalamins, it was necessary to evaluate the action of methylcobalamin on processes of growth of various types of tumors.

The stimulating influence of cyanocobalamin on the growth and development of certain transplantable (sarcoma 45, Guerin carcinoma, Walker carcinosarcoma, sarcoma 180, Lewis sarcoma, etc.) and induced tumors is evidently due to its conversion to cobalamin coenzymes in the animal organism. Methylcobalamin and adenosylcobalamin have been detected in spleen cells of mice with La Leukemia, as well as in leukemia L-1210 and Ehrlich's ascites carcinoma cells [8-10].

The aggregate of clinico-experimental data thus determined the advisability of the search for effective antagonists of cobalamins for the blocking of certain cobalamin-dependent reactions. In view of the activating influence of methylcobalamin on methionine synthetase and the increase in the total pool of tetrahydrofolic acid (THFA) in the cells, regardless of the folate reductase system, the greatest attention is attracted by antagonists of methylcobalamin [11-13]. In our investigations using methylcobalamin antagonists to lower the methionine synthetase activity, we succeeded in slowing down the processes of growth of bacterial and embryonic cells, as well as certain types of tumors [6, 14]. It was also shown that the antineoplastic activity of methotrexate – a specific inhibitor of folate reductase – increases when it is used in combination with methionine synthetase inhibitors [14].

In addition, there is still another possibility of enhancing the antineoplastic activity of methotrexate with cobalamins. The prerequisite for this means of combined influence with methotrexate was experimental data showing the ability of cobalamins to stimulate processes of proliferation and to increase the number of DNA-synthesizing cells, most sensitive to methotrexate, in the population [15, 4].

The present communication presents the results of the combined action of methylcobalamin and methotrexate on various transplantable tumors in animals.

EXPERIMENTAL

The experiments were conducted on mice of the $C_{57}BL$, CBA, and BALB/c lines, the hybrids BDF_1-($C_{57}BL \times DBA/2$), $F_1(C_{57}BL \times CBA)$ and SHK mice, obtained from the nursery of the Academy of Medical Sciences of the USSR. In the experiments we used 420 mice, weighing 20-25 g.

The action of methylcobalamin was studied on solid tumors: adenocarcinoma of the mammary gland (Ca-755), cancer of the cervix (RShM-5), adenocarcinoma of the intestine (AKATOL), sarcoma 37, as well as on leukemia L-1210, according to the procedure used in the laboratory [13, 14].

Methylcobalamin (CH_3Cbl), synthesized according to the method of [17], was injected intramuscularly in doses of 10 and 500 $\mu g/kg$ twice at 96-h intervals or 500 $\mu g/kg$ daily for five days.

Methotrexate (from Lederle) was used in a dose of 10 mg/kg intraperitoneally twice at a 96-h interval.

In part of the experiments, methylcobalamin chloropalladate ($CH_3Cbl \cdot PdCl_2$; I) and dibromide-4-[[[[[1-methylpyridino-4-amino]phenyl]amino]carbonyl]phenyl]amino]-8-amino-1-methylquinoline (NSC-176319; II), which we obtained from the National Cancer Institute of the United States according to the program of cooperation between the USSR and the US in the field of chemotherapy of tumors [18], were used as methionine synthetase inhibitors.

Complex I, synthesized at the All-Union Vitamin Scientific Research Institute [19], was administered perorally in a dose of 250 mg/kg; the quinolinium derivative II was administered intraperitoneally in a dose of 5 mg/kg twice at a 96-h interval.

The treatment of the animals was begun 48 h after transplantation of the tumor. The antineoplastic effect was estimated directly after the end of the course of therapy and at various periods over the subsequent life of the animals.

The criteria of effectiveness were the percent inhibition of tumor growth, calculated according to its volume, and the increase in the lifetime of the animals. The data obtained were subjected to statistical treatment according to the Student method.

RESULTS AND DISCUSSION

From the data that we obtained it follows that methylcobalamin substantially stimulates the growth of transplantable tumors: Ca-755, AKATOL, and to a lesser degree RShM-5 and sarcoma 37 (Table 1).

The intensity of tumor growth depended on the line of experimental animals, the frequency of administration, and the concentration of methylcobalamin. The greatest stimulating effect on growth of the tumor Ca-755 was noted in the case of two administrations of the preparation in a dose of 10 $\mu g/kg$ after transplantation of the tumor into the hybrids BDF_1 (+180%), and to a lesser degree for mice of the pure line $C_{57}BL$ (+75%). In F_1 hybrids, a substantial intensification of tumor growth was detected in the case of five administrations of methylcobalamin in a dose of 500 $\mu g/kg$. The stimulation of the growth of Ca-755 and AKATOL was followed for a period of two to three weeks, whereas in mice with sarcoma 37 and RShM-5, it was noted only directly after the end of the course of administration of the preparation. In mice of the pure line ($C_{57}BL$), intensified tumor growth was observed for a longer period (2-3 weeks after transplantation of the tumor) than in hybrids. For precisely this reason, in subsequent investigations of the action of methylcobalamin and its analogs on the cell kinetics of Ca-755, we used mice of the $C_{57}BL$ line.

In the case of simultaneous administration of methotrexate and methylcobalamin, an intensification of their inhibiting effect on tumor growth was observed (L-1210, Ca-755, RShM-5). The lifetime of animals with leukemia L-1210 was increased by 78% in this case, whereas in the case of isolated administration of methotrexate the increase was only 55%. The most rapid results were obtained for adenocarcinoma of the mammary gland (Table 2). In this case the combination of methotrexate with methylcobalamin increased the lifetime of the animals by 60%, which was three times as great as the effect of methotrexate alone. On the 8th to 14th days after the end of the combined course of therapy with methylcobalamin and methotrexate, the inhibition of tumor growth was 76-40%, respectively, whereas methotrexate alone had practically no activity at the same periods (31-0%).

It is known that as solid tumors grow, the number of cells in the resting phase in them increases substantially, and the sensitivity of the tumors to cyclospecific preparations decreases appreciably [20]. Evidently the sensitivity of the tumor to methotrexate can be substantially increased by administering methylcobalamin,

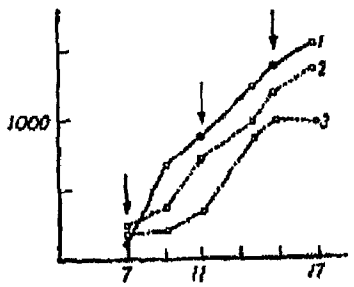

Fig. 1

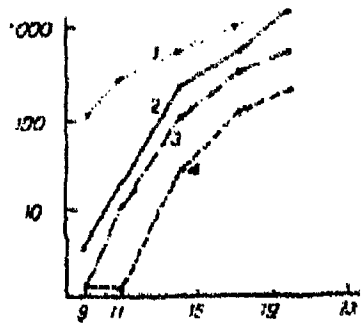

Fig. 2

Fig. 1. Combined action of methylcobalamin and methotrexate on growth of RShM-5. Along x-axis: period after administration of preparation (in days); along y-axis: average volume of tumor (in mm^3). The arrows indicate the time of administration of the preparations. 1) Control; 2) methotrexate; 3) methylcobalamin + methotrexate.

Fig. 2. Combined action of methylcobalamin, methionine synthetase inhibitors, and methotrexate on growth of Ca-755. Along x-axis: periods after administration of preparations (in days); along y-axis: average volume of tumor (in mm^3). 1) Control; 2) methotrexate; 3) $CH_3Cbl + NSC-176319 + CH_3Cbl \cdot PdCl_2$ + methotrexate; 4) $NSC-176319 + CH_3Cbl \cdot PdCl_2$ + methotrexate.

which increases the total pool of DNA-synthesizing cells. To test this hypothesis we used transplantable mouse cancer of the cervix (RShM-5). This tumor is characterized by slow growth, which makes it possible to administer a longer course of therapy and to evaluate the antineoplastic effect in long-term periods after transplantation. The experimental results confirmed our hypothesis. The average volume of the tumor in the control group of animals on the 11th day after transplantation of RShM-5 exceeded the initial volume, when treatment of the animals was begun (7th day after transplantation), by 5.2-fold. In animals that received only methotrexate, the volume of the tumor at the same periods was increased 3.5-fold, and in the case of joint administration with methylcobalamin, there was only a 1.7-fold increase. Repeated combined administrations of the preparations (each four days) led to an inhibition of tumor growth in longer term periods as well. Thus, on the 17th day after transplantation, the average volume of the tumors in the control group exceeded the original volume by 22.7-fold, in the group of animals treated with methotrexate by 12.7-fold, and in the group of mice that received methylcobalamin and methotrexate by 7.6-fold (Fig. 1).

The interval between administration of methylcobalamin and methotrexate is of vital importance. According to the data obtained, with increasing time between administrations of the preparations, a partial or total loss of activity of methotrexate is noted, and in certain cases even the appearance of an effect of stimulation (see Table 2). Thus, for example, the inhibition of growth of Ca-755 on the 7th day after the end of treatment with methotrexate was 69%. And yet, when methylcobalamin was preliminarily administered (6 h before the use of methotrexate), a total loss of activity of methotrexate was observed. The weakening of the antineoplastic activity of methotrexate is especially pronounced in the hybrids BDF_1. As was shown, precisely in mice of this line, methylcobalamin induced the greatest stimulation of tumor growth. In F_1 hybrids with the absence of a stimulating effect, in the case of its isolated use, the combined influence did not lead to any weakening of the methotrexate activity. An appreciable decrease in the antineoplastic activity of methotrexate after preliminary administration of methylcobalamin is evidently due to activation of the cobalamin-dependent methionine synthetase system and an increase in the total pool of the TGFA of the cells. This is confirmed by the results of the combined action of methotrexate and inhibitors of methionine synthetase against a background of preliminarily administered methylcobalamin (Fig. 2). The joint influence of methylcobalamin chloropalladate, the quinolinium derivative, and methotrexate substantially exceeds the activity of a combination of the same preparations with methylcobalamin. Thus, the inhibition of growth of Ca-755 on the 14th day after the end of therapy of mice that received methotrexate and complexes II and I was 86%, whereas in the case of combined influence of the three inhibitors with methylcobalamin it was only 61%. The increase in the lifetime of the animals in these groups was 30% ($P < 0.05$) and 15% ($P > 0.05$), respectively.

Thus, in our investigations the stimulating action of methylcobalamin on the growth of certain solid tumors in mice was demonstrated for the first time. The results of our investigations permit an explanation of the decrease in the therapeutic effect of a number of alkylating preparations (sarcolysin, thioTEPA, embitol, and novembitol) in the case of their simultaneous use with cyanocobalamin [21-23]. The stimulating effect of methylcobalamin on solid tumors is clearly correlated with recent investigations, in which a significant increase in the methylcobalamin content in the rat liver was revealed after the administration of a chemical carcinogen and in certain transplantable Morris hepatomas [24]. It is important to note that the frequency of development of hemoblastoses of mice and of the simultaneous influence of methylcobalamin with endogenous blastomogens also increases significantly [25]. The aggregate of the indicated experimental data thereby confirms the involvement of methylcobalamin in processes of proliferation of tumor cells of various histogenesis.

In discussing the mechanism of the combined action of methylcobalamin with methotrexate, in our opinion, we should consider two possible aspects. In view of the fact that cobalamins promote the entry of the basic transport form of folic acid (methyl-THFA) into cells, and there is a common pathway of active transport of methyl-THFA and methotrexate into cells [26, 27], it can be assumed that methylcobalamin also influences the transport of methotrexate. At present there are no data in the literature on the mechanism of the penetration of methotrexate into Ca-755 cells. However, under our conditions of influence, at a physiological level of methyl-THFA in the blood of the animals and the therapeutic concentration of methotrexate, evidently the possibility of facilitated penetration of the latter into the tumor cells is realistic.

Vitally important factors in the combined influence are activation of the methionine synthetase reaction and an increase in the number of DNA-synthesizing tumor cells, i.e., those most sensitive to methotrexate, under the influence of methylcobalamin. This may play a deciding role in the increase in the antineoplastic activity of methotrexate when it is administered simultaneously with methylcobalamin. The data that we obtained at present on the study of the cell kinetics of Ca-755 under the influence of methylcobalamin confirm this premise.

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DERIVATIVES OF ARYLSULFONIC ACIDS. SYNTHESIS AND HYPOGLYCEMIC ACTIVITY OF 4-ALKOXYBENZENESULFONYLCARBAMIDES AND 4-ALKOXYBENZENESULFONYLTHIOSEMICARBAZIDES

M. A. Kaldrikyan, V. A. Geboyan, A. A. Aroyan,* N. O. Stepanyan, and Zh. M. Buniatyan

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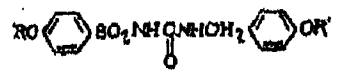
In order to find a link between the chemical structure and the hypoglycemic activity of 4-alkoxybenzenesulfonic acids [1], we have prepared some alkoxybenzenesulfonylcarbamides I and sulfonylthiosemicarbazides II.



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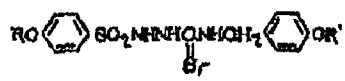
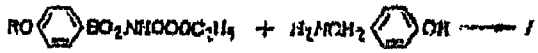


Compounds I and II differ from those synthesized earlier [2] in that they contain an alkoxybenzyl radical so that the effect of this group on the hypoglycemic activity can be determined.

One of the most suitable and convenient methods of synthesizing I [3] is by the reaction of the ethyl esters of 4-alkoxybenzenesulfonylcarbamic acids with alkoxybenzylamines:



The ethyl 4-alkoxybenzenesulfonylcarbamates were prepared by heating 4-alkoxybenzenesulfonylamides with ethyl chlorocarbonate in the presence of dry potash [4]. The 4-alkoxybenzylamines were synthesized by the condensation of alkoxybenzyl chlorides with potassium phthalimide with subsequent hydrolysis of the alkoxybenzylphthalimides [5].

Structures of the 4-alkoxybenzenesulfonylcarbamides I were confirmed by IR spectroscopy; the SO_2 group gives rise to absorption bands at 1170 and 1370, 1180 and 1335, 1185 and 1340 cm^{-1}, and the associated NH group gives characteristic bands at 3240-3380 cm^{-1}.

The sulfonylthiosemicarbazides II were prepared by heating 4-alkoxybenzenesulfonylhydrazides [1] and 4-alkoxybenzeneisothiocyanates [6] for two h:

In the IR spectra of the latter, the C=S group absorbs at 1565, 1180, and 975 cm^{-1}, the SO_2 group at 1160 and 1330 cm^{-1}, and 1175 and 1340 cm^{-1}, and the NH group at 3160, 3265, and 3370 cm^{-1}.

*Deceased.

A. L. Mndzhoyan Institute of Fine Organic Chemistry, Erevan. Translated from Khimiko-Farmatsevticheskii Zhurnal, Vol. 12, No. 10, pp. 54-57, October, 1978. Original article submitted January 13, 1978.

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Всесоюзный онкологический научный центр АМН СССР, Москва

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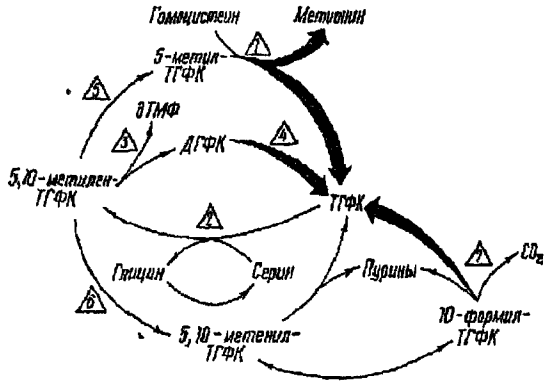
Противоопухолевая эффективность метотрексата при его комбинированном применении с кобаламиновыми производными

Н. В. МЯСИЩЕВА, З. П. СОФЬИНА, О. Д. ГОЛЕНКО, Ф. Г. АРСЕНЯН

Клинико-экспериментальные исследования показали, что один из коферментов витамина B_{12}— метилкобаламин в определенных условиях проявляет свойства, характерные для модифицирующих факторов канцерогенеза [1]. В частности, канцерогенная активность некоторых метаболитов триптофана и тирозина, включая пара-оксифенилмолочную кислоту, в организме животных возрастает при одновременном введении с кобаламиновым коферментом. Воздействие метилкобаламина приводит к значительному сокращению латентного периода при возникновении индуцированных гемобластозов и существенно повышает частоту их развития [2—4]. Метилкобаламин оказывает также стимулирующее действие на рост перевиваемых опухолей [5]. Высокая биологическая активность метилкобаламина в основном обусловлена его ролью кофермента метионинсинтетазы (К.Ф.2.1.1.13), контролирующей в клетках млекопитающих метаболизм фолата [6, 7]. Завершающий этап биосинтеза метионина составляет главный пусковой механизм цикла фолатзависимых реакций в процессе роста клеток при образовании пуринов и пирамидинов [8] (рисунок).

Нормальный баланс между свободной тетрагидрофолиевой кислотой (ТГФК) и ее одноуглеродистыми производными зависит от интенсивности синтеза пуринов и пиримидинов, а также активности метионинсинтетазы и дигидрофолатредуктазы. При увеличении концентрации метионина возрастает активность формил-ТГФК дигидрогеназы. С помощью этого звена в клетках регулируется не только обмен фолатов, но и самой незаменимой аминокислоты. Последнее необходимо для предоставления адекватного количества метионина для синтеза полиаминов и белка [8]. Путем обратного торможения метилен-ТГФК редуктазы метионин контролирует также содержание метил-ТГФК. Высокий уровень последней сни-



Цикл фолиевой кислоты и сопряженных процессов биосинтеза метионина, пуринов и пиримидинов:

1 — кобаламинзависимая метионинсинтетаза, *2* — серинокси-метилтрансфераза, *3* — тимидилатсинтетаза, *4* — дигидрофолатредуктаза, *5* — метилен-ТГФК редуктаза, *6* — метилен-ТГФК дегидрогеназа, *7* — формил-ТГФК дегидрогеназа.

жает активность серинокси-метилтрансферазы, необходимой для образования метилен-ТГФК и последующего формирования пиримидинов совместно с тимидилатсинтетазой. При участии метилен-ТГФК дегидрогеназы осуществляется также образование 5, 10-метенил- и формил-ТГФК для синтеза пуринов. Превращение основной транспортной формы фолиевой кислоты — метил-ТГФК в коферменты (метилен- и формил-ТГФК) лимитирует кобаламинзависимая метионинсинтетаза. Нарушение синтеза ДНК в кроветворных клетках человека в результате «ловушки метил-ТГФК», снижения концентрации свободной ТГФК и ее коферментов при недостаточности витамина B_{12} подтверждает значимость в этом процессе метилкобаламина [9, 10]. Вместе с тем его высокое содержание в сыворотке крови больных острым лейкозом является, по-видимому, одной из причин их малой чувствительности к комбинированной химиотерапии благодаря развитию кобаламинзависимого спасательного пути в опухолевых клетках [11].

Наши экспериментальные данные о модифицирующем действии кобаламинов на процессы роста перевиваемых и индуцируемых опухолей в организме животных обосновали реальную возможность использования кобаламиновых производных для повышения эффективности химиотерапии. В данных исследованиях основное внимание уделяли анализу действия метилкобаламина и его антагонистов на противоопухолевую активность метотрексата при перевиваемых солидных опухолях животных.

Методика исследований. Опыты проведены на 420 мышах линии C57Bl/6, BALB/c, F_1, BDF (C57Bl×ДВА/2), F_3 (C57Bl×CBA), а также на мышах SHK, полученных из питомника АМН СССР. Нами использованы следующие модели перевиваемых солидных опухолей: аденокарцинома молочной железы (Са-755), рак шейки матки (РШМ-5), аденокарцинома толстого кишечника (АКАТОЛ), рак легкого Льюис (LLC) и саркома 37 (С-37). Химиотерапевтические опыты проведены в соответствии с ранее опубликованной схемой [5]. В разных сериях исследовано влияние метилкобаламина, который вводили внутримышечно на 3-и и 7-е сутки после перевивки опухолей и двух его антагонистов: хлордифтор- и хлорпалладата метилкобаламина. Препараты вводили в дозе 250 мг/кг массы ежедневно в течение 5 суток, первый — внутримышечно, а второй — перорально. Кобаламиновые производные были синтезированы в научно-производственном объединении «Витамины». Использовали также метотрексат («Lederle», США). Результаты действия препаратов на солидные опухоли оценивали непосредственно через 24 ч после курса лечения и в отдаленные сроки. Критерием эффективности при этом служили процент стимуляции и торможения роста опухоли, вычисляемые по условному объему $\left(\frac{v_0 - v_k}{v_k} \cdot 100\ \%\right)$, и увеличение продолжительности жизни животных. Пролиферативную активность опухолевых клеток исследовали с помощью метода авторадиографии с ^{3}H-тимидином.

Результаты исследований и их обсуждение. Установлено стимулирующее действие метилкобаламина на рост перевиваемых солидных опухолей. Введение малых доз метилкобаламина значительно ускоряет рост Са-755 и АКАТОЛ. Не столь выраженное и более кратковременное стимулирующее действие метилкобаламина выявлено при РШМ-5 и С-37 (табл. 1). В минимальной дозе (0,01 мг/кг массы) метилкобаламин усиливал рост Са-755 незначительно и на 5-е сутки после его введения объем опухоли превышал контроль лишь на 56 %. При двукратном введении метилкобаламина (суммарная доза 0,02 мг/кг) на 7-е сутки роста опухоли объем ее увеличивался в 2,3—2,8 раза по сравнению с контролем. В последующие 2—3 недели рост опухоли замедлялся. Как показано ранее [12], степень и продолжительность стимуляции роста Са-755 возрастали с увеличением дозы вводимого метилкобаламина и зависели от линии мышей. Специфичность действия метилкобаламина на процессы роста опухоли подтверждают результаты сравнительной оценки активности двух кобаламиновых коферментов. Согласно нашим данным, в отличие от метилкобаламина введение животным аналогичной дозы 5-дезоксиаденозилкобаламина (К. Ф. 5. 4. 99. 2) практически не влияло на

Таблица 1. Влияние метилкобаламина на рост перевиваемых опухолей мышей

Опухоль	Доза препарата, мг/кг	Объем опухоли, % к контролю	
		Сроки после окончания введения препарата, сутки	
		1—2	7—8
Са-755	0,01	180	65
АКАТОЛ	0,01	126	37
РШМ-5	0,01	47	0
С-37	0,50	57	0

Примечание. Метилкобаламин вводили на 3-и и 7-е сутки роста опухоли. Результаты статистически достоверны ($P<0,05$) по отношению к контролю.

рост Са-755. Стимулирующее действие метилкобаламина на рост опухолей определило целесообразность использования некоторых его антагонистов для торможения кобаламинзависимых реакций в организме животных. В качестве потенциальных противоопухолевых соединений нами были исследованы хлордифторметилкобаламин и хлорпалладат метилкобаламина. Эти производные метилкобаламина тормозили в культуре бласттрансформированных лимфоцитов крови человека поступление в клетки предшественника кобаламиновых коферментов и их биосинтез [13]. Из указанных аналогов метилкобаламина бо́льшую противоопухолевую активность проявил хлорпалладат метилкобаламина. В зависимости от схемы введения препарат тормозил рост Са-755, РМШ-5 и LLC на 70—80 %. При аналогичном режиме введения хлордифторметилкобаламин не проявил выраженной активности, что подтверждало наши данные, полученные при исследовании in vitro. По-видимому, это обусловлено происходящим торможением биосинтеза аденозилкобаламина в опухолевых клетках.

Для обоснованного применения кобаламинов в комбинированной химиотерапии опухолей важное значение имело изучение различных аспектов их действия на организм животных с перевиваемыми опухолями. С этой целью на модели Са-755, наиболее чувствительной к их воздействию, мы исследовали in vivo основные параметры пролиферации клеток, биосинтез кобаламиновых коферментов и активность кобаламинзависимой метионинсинтетазы в опухолевых клетках. Согласно полученным ранее данным, при введении кобаламинового кофермента время генерации (T_c) и его отдельных периодов (t_s; t_{g2}; $t_{g1}+t_m$) клеток Са-755 не изменяется и составляет соответственно 12, 6, 2, 4 ч [8, 14]. При воздействии метилкобаламина в опухоли существенно увеличивается количество пролиферирующих клеток. Следует отметить, что фактор потери клеток в опухоли минимален и возрастает несущественно при воздействии кобаламинового кофермента. Статистически значимые различия индекса меченных ^{3}H-тимидином клеток отмечаются в Са-755 мышей через 24 ч после введения метилкобаламина. В экспоненциальной фазе роста опухоли при воздействии метилкобаламина индекс метки увеличивается в 1,4 раза по сравнению с контролем. Аналогичные результаты получены нами при многократном введении ^{3}H-тимидина. Величина индекса метки в опухоли при воздействии метилкобаламина была существенно выше контроля (56,9±2,1 % и 42,8±1,3 % соответственно). Значение пролиферативного пула, рассчитанное методом сравнения наблюдаемого и ожидаемого индексов метки, также было значительно повышено при введении метилкобаламина.

Известно, что в процессе роста большинства со́лидных опухолей животных пул пролиферирующих клеток уменьшается. В Са-755 на 6—14-е сутки роста количество меченых клеток после однократного введения ^{3}H-тимидина постепенно снижается с 28 до 9 %. При воздействии метилкобаламина в процессе роста Са-755 также наблюдается уменьшение индекса метки. Однако при этом повышенное количество меченых клеток в опухоли сохраняется и в поздних стадиях роста. Таким образом, при введении небольшой дозы метилкобаламина наблюдается существенное увеличение пула пролиферирующих клеток, наиболее чувствительных к ингибирующему действию циклоспецифических веществ. При исследованиях кинетики роста и пролиферации клеток Са-755 мы оценивали также действие хлорпалладата метилкобаламина. При его введении животным торможение роста опухолей отмечается в ранней экспоненциальной фазе [15]. Показатели пула пролиферирующих клеток в опухоли через 48 ч после воздействия хлорпалладата метилкобаламина уже существенно не отличаются от их значений в контроле.

Совокупность полученных нами данных позволила сформулировать принципиально новый подход к комбинированной химиотерапии опухолей на основе использования модифицирующего влияния кобаламинов на процессы их роста. Учитывая синергизм действия соединений фолиевой кислоты и кобаламинов в процессах пролиферации клеток, представлялось возможным существенно повысить противоопухолевую активность метотрексата. Мы исследовали альтернативные пути возрастания противоопухолевой активности метотрексата при его комбинированном применении с кобаламиновыми производными. Повышения избирательности действия метотрексата удалось достигнуть в результате увеличения в опухоли пула пролиферирующих клеток с помощью метилкобаламина. Значительное увеличение противоопухолевой активности метотрексата наблюдалось при его сочетанном применении с метилкобаламином у животных с Са-755, РШМ-5 и лейкозом L 1210. Противоопухолевый эффект метотрексата — специфического ингибитора дигидрофолатредуктазы заметно возрастает также при одновременном блокировании в опухолевых клетках метионинсинтетазы с помощью антагониста метилкобаламина или производного хинолина Кейна [16, 17]. Следует отметить, что противоопухолевая активность исследованных антагонистов метилкобаламина незначительна и не обеспечивает длительного торможения роста опухоли. Однако ограничение в клетках спасательного пути образования фолиевых коферментов создает достаточный фон для увеличения противоопухолевого действия метотрексата (см. рисунок). При его комбинированном

Таблица 2. Комбинированное действие метотрексата, кобаламиновых производных и хинолина Кейна на рост Са-755 мышей линии С57Bl

Препарат	Доза препарата, мг/кг	Торможение роста опухоли, % к контролю			Увеличение продолжительности жизни животных, % к контролю
		Сроки после введения препарата, сутки			
		1—2	7—8	14—15	
Метотрексат	10	75	44	18	0
Метотрексат+ +метилкобаламин	10+0,01	99	70	9*	19*
Метотрексат+ +хлорпалладат метилкобаламина	10+250	98	88	—	0
Метотрексат+ +хлорпалладат метилкобаламина+ +хинолин Кейна	10+ +250+5	100	96	85	30

Примечание. Препараты вводили на 3-и сутки роста опухоли; метотрексат и хинолин Кейна — внутрибрюшинно двукратно с интервалом 96 ч; хлорпалладат метилкобаламина — пятикратно с интервалом 24 ч. * $p > 0{,}05$

применении с хлорпалладатом метилкобаламина торможение роста Са-755, РШМ-5, АКАТОЛ существенно возрастает и проявляется в сроки, когда активность одного метотрексата практически отсутствует (табл. 2).

В механизме стимулирующего действия метилкобаламина важным является его способность индуцировать активность метионинсинтетазы в опухолевых клетках. Это продемонстрировано ранее в культуре клеток млекопитающих и опухолевых клетках человека. Однако не все виды опухолевых клеток могут осуществлять in vitro биосинтез метионина, необходимый для их роста [18]. В связи с этим заслуживают внимания наши экспериментальные данные о возрастании активности метионинсинтетазы в клетках Са-755 при введении животным метилкобаламина [15]. Очевидно, снижение количества холофермента при воздействии хлорпалладата метилкобаламина обусловлено меньшим поступлением в опухолевые клетки активных форм кобаламинов в результате конкуренции за транспортный белок — транскобаламин II. Наши экспериментальные данные подтверждают, что характерное накопление опухолью циан-Co^{57} кобаламина в экспоненциальной фазе роста Са-755 и формирование в клетках меченых кобаламиновых коферментов резко снижаются при введении животным антагониста метилкобаламина [19].

Усиление противоопухолевой активности метотрексата при одновременном применении с метилкобаламином, как мы полагаем, в значительной степени зависит от возможности торможения синтеза ДНК в бо́льшей части популяции опухолевых клеток. И хотя не исключено влияние метилкобаламина на скорость поступления метотрексата в опухолевые клетки [17], выяснение этого вопроса требует специальных исследований. В настоящее время мы располагаем новыми экспериментальными доказательствами повышения с помощью метилкобаламина противоопухолевой активности и другого S-фазовоспецифичного антиметаболита — арабинозидцитозина.

N. V. Myasishcheva, Z. P. Sofina, O. D. Golenko, F. G. Arsenyan

ANTITUMOUR EFFICIENCY OF METHOTREXATE IN COMBINATION WITH COBALAMINE DERIVATIVES

Summary

Improvement of the antitumour activity of methotrexate is suggested on the basis of modifying action of cobalamine on processes of the tumour growth. The influence of methylcobalamine and two of its analogues is shown on various experimental models: Ca—755, Ca cervix uteri and colon, LLC, sarcoma 37 of mice.

Two alternative ways to increase the therapeutic efficiency of methotrexate are analyzed with due regard for kinetic parameters of cell proliferation, of Cbl-coenzymes biosynthesis and activity of Cbl-dependent methionine synthetase in the tumour.

All-Union Cancer Research Centre,
Academy of Medical Sciences, USSR, Moscow

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Всесоюзный онкологический научный центр АМН СССР, Москва

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Молекулярные механизмы регуляции роста опухолей молочной железы эстрогенами

Т. М. МОРОЗОВА, Т. И. МЕРКУЛОВА, Р. И. САЛГАНИК

Известно, что эстрогены в тканях-мишенях (матке, молочной железе) активируют пролиферацию эпителиальных тканей; этому предшествует индукция синтеза РНК и белков-ферментов, обеспечивающих последующий синтез ДНК и митозы. Нередко под контролем эстрогенов находится также и рост опухолей молочной железы (ОМЖ) экспериментальных животных и человека. В таких опухолях, как и в нормальных тканях, эстрадиол стимулирует синтез РНК, белков, ДНК и активирует клеточное деление. Однако часть опухолей в ходе малигнизации теряет способность реагировать на эстрадиол; такие опухоли становятся эстрадиолнезависимыми.

Изучение молекулярных механизмов взаимодействия эстрадиола с клетками опухолей позволяет выяснить причины утраты зависимости роста опухолей от эстрогенов, что имеет не только теоретическое значение, так как для определения правильной тактики лечения больных с ОМЖ и для обоснованного применения эндокринной терапии необходимо установить, зависит ли рост данной опухоли от эстрогенов. В настоящее время нет достаточно надежных способов дискриминации эстрогензависимых и эстрогеннезависимых ОМЖ. Создание таких способов возможно на основании знания молекулярных механизмов действия эстрадиола на опухолевые клетки. Нашей задачей было изучение взаимодействия эстрадиола с эстрогензависимыми и эстрогеннезависимыми опухолями, выяснение механизмов нарушений в нем для того, чтобы на этой основе разработать критерии оценки эстрогензависимости опухолей.

Опыты проводили на ОМЖ мышей высокораковых линий DD и C3H и на индуцированных 7,12-диметилбензантраценом (ДМБА) ОМЖ крыс линии Sprague-Dawley. Об эстрогензависимости опухолей судили по влиянию на их рост овариоэктомии животных и введения им эстрадиола [1]. Эстрогензависимыми считали опухоли, которые регрессировали после овариоэктомии. Введение эстрадиола стимулировало рост этих опухолей. Были выделены эстрогеннезависимые опухоли, на рост которых не влияли ни овариоэктомия, ни введение эстрадиола. Для выяснения причин утраты зависимости роста опухолей от эстрогенов прежде всего исследовали содержание рецепторов эстрадиола в клетках эстрогензависимых и эстрогеннезависимых опухолей (рис. 1), которое определяли по опи-

Myasishcheva N.V., Sofyina Z.P., Golenko O.D., Arnesyan F.G.

Antitumor activity of methotrexate when used in combination with cobalamine derivatives

Clinical and experimental studies have shown that one of the coenzymes of vitamin B12 – methylcobalamine – has the qualities common for the modifying factors of oncogenesis under some conditions [1]. In particular, the oncogenic activity of some metabolites of tryptophane and tyrosine, including para-oxy-phenyl-lactic acid, increases with the simultaneous administration of cobalamine coenzyme in the organism of animals.

Methylcobalamine's effect leads to a significant reduction of the latent period in the development of induced hemoblastoses and increases the frequency of their progression [2-4]. Methylcobalamine also has a stimulating effect on the growth of subinoculated tumors [5]. The high biologic activity of methylcobalamine can be explained by its role as a coenzyme for methionine synthase (2.1.1.13), which controls folate metabolism in the cells of mammals [6, 7]. The final stage of methionine biosynthesis is the main starting mechanism in the cycle of folate-dependent reactions in cell proliferation during formation of purine and pyrimidine [8] (Picture).

The normal balance between unbound tetrahydrofolic acid (THFA) and its single-carbon derivatives depends on the activity of purine and pyrimidine synthesis and also on the activity of methionine synthase and dihydrofolate-reductase. An increase in methionine concentration leads to an increase in formyl-THFA-dihydrogenase. This is the key regulating point not only for folate metabolism, but also for the metabolism of essential amino acid itself. The latter is necessary to allow an adequate methionine concentration for polyamine and protein synthesis [8]. Methionine also controls the concentration of methyl-THFA by means of feedback inhibition of methylene-THFA reductase. The high level of the latter reduces the activity of serinoxymethyltransferase that is necessary for methylene-THFA formation and consequent formation of pyrimidines with thymidylate synthase. Methylene-THFA dehydrogenase is also essential for 5, 10-methenyl- and formyl-THFA formation for purine synthesis. Cobalamine-dependent methionine synthase limits the transformation of the main transport type of folic acid – methyl-THFA – to coenzymes (methylene- and formyl-THFA). The disturbance of DNA

synthesis in the hematopoietic cells of a human due to "methyl-THFA trap", decrease of free THFA and its concentration of coenzymes that lacks a sufficient amount of vitamin B12 confirm the importance of methylcobalamine in this process [9, 10]. In addition, its high concentration in the blood serum of the patients with acute leucosis is, probably, one of the reasons of low sensitivity of these patients to the combined chemotherapy due to the formation of cobalamine-dependent protection pathway in tumor cells [11].

Our experimental data on the modifying effect of cobalamines on the growth processes of subinoculated and induced tumors in animals proves the possibility for use of cobalamine derivatives to increase the effectiveness of chemotherapy. This data was mostly concerned with analysis of the effect of methylcobalamine and its antagonists on the antitumor activity of methotrexate in subinoculated solid tumors of animals.

Research Method

Experiments were carried out on 420 mice of the lines $C_{57}B\frac{1}{6}$, BALB/c, F_1 BDF ($C_{57}B_1$XDBA/2), F_3 ($C_{57}B_1$XCBA), and on mice SHK, delivered from the nursery of the Academy for Medical Sciences, USSR. We used the following types of subinoculated solid tumors: breast adenocarcinoma (Ca-755), cervical cancer (CC-5), large intestine adenocarcinoma (ACALI), Lewis lung cancer (LLC) and sarcoma 37 (C-37). Chemotherapeutic experiments were carried out according to the previously published protocol [5]. The effect of methylcobalamine that was administered intramuscularly 3 and 7 days after the subinoculation of the tumors as well as the effect of its 2 antagonists: chloridedifluoride- and methylcobalamine chloride palladate was evaluated. 250mg/kg (of body weight) of drugs were administered daily for 5 days, first – intramuscularly, second – per os. Cobalamine derivatives were synthesized in the Scientific center "Vitamins". Methotrexate (company "Lederle", USA) was also used. The effect of the drugs on solid tumors was evaluated 24 hours after the treatment and later. The percentage of the tumor growth stimulation and reduction that were measured based on specific volume $\left(\frac{v_0 - v_k}{v_k} \cdot 100\%\right)$ and the increase in the life span of animals were chosen as criteria for effectiveness. The proliferative activity of the tumor cells was evaluated with autoradiography with H^3 tymidine method.

Study Results and their discussion

The stimulating effect of methylcobalamine on the growth of subinoculated solid tumors has been established. The administration of small doses of methylcobalamine significantly increases the proliferation of Ca-755 and ACALI. However, the effect of methylcobalamine on cervical cancer-5 and C-37 was not as expressed and more short-acting (Table 1). A minimal dose of methylcobalamine (0.01 mg/kg) insignificantly increased the proliferation in Ca-755, and on the 5^{th} day after its administration, the tumor was only 56% bigger in size than that of the control group. If administered twice a day (0.02 mg/kg), methylcobalamine caused 2.3-2.8-fold increase in the tumor growth on the 7^{th} day in comparison with the control group. The proliferation slowed down in the following 2-3 weeks. As has been shown earlier [12], the level and length of the growth stimulation of Ca-755 increased with the increase of administered methylcobalamine and were dependent on the line of the mice. The specificity of the methylcobalamine effect on the proliferation processes of tumor confirms the comparative evaluation results of the effect of two cobalamine coenzymes. According to our data, unlike cobalamine, the administration of the same dose of 5-desoxypadenosilcobalamine did not alter the growth of Ca-755. The stimulating effect of methylcobalamine on the growth of tumor determined the practicability for the usage of some of its antagonists in order to reduce the activity of cobalamine-dependent reactions in the organism of animals. We investigated chloridedifluoridemethylcobalamine and methylcobalamine chloride palladate as potential antitumor compounds. These derivatives of methylcobalamine slowed the entry of precursors of cobalamine coenzymes and their biosynthesis in the culture of blast-transformed blood lymphocytes in human blood [13]. Out of the two derivatives mentioned above, methylcobalamine chloride palladate had a higher antitumor activity. Depending on the administration scheme, this drug showed a 70-80% reduction of the proliferation in Ca-755, CC-5 and LLC. Using the same method of administration, chloridedifluoridecobalamine did not show such a pronounced activity, which confirmed the data that we received from the in vitro studies. It can probably be explained by the suppression of adenosilcobalamine biosynthesis in the tumor cells.

To use cobalamines in the combined tumor chemotherapy, it is important to study the different aspects of its action on the organisms of animals with subinoculated tumors. Therefore, we researched the main parameters of cellular proliferation, biosynthesis of cobalamine coenzymes and activity of cobalamine-dependent methionine synthase in vivo in the tumor cells of the mice with Ca-755, since they proved to be the most sensitive to the activity of cobalamine. According

to the data obtained earlier, the administration of cobalamine coenzyme does not change the generation time (T_c) and its separate periods (t_s; t_{g2}; $t_{g1}+t_m$) of the cells Ca-755 and constitutes 12, 6, 2, 4 hours correspondingly [8, 14]. The amount of the proliferating cells in the tumor increases significantly with the activity of methylcobalamine. It should be noted that the factor of cellular loss in tumor is minimal and increases insignificantly under the effect of cobalamine coenzyme. A statistically important difference in the index of the cells marked with ^{3}H-thymidine is noticed in Ca-755 of the mice 24 hours after the administration of methylcobalamine. The exponential phase of the tumor growth under the effect of methylcobalamine shows a 1.4 times increase compared to the control group. Analogous results were seen in multiple administrations of ^{3}H-thymidine. The index of the marker in the tumor under the effect of methylcobalamine was much higher than that in the control group (56.9+2.1% and 42.8 ±1.3% correspondingly). The proliferating pool value that was calculated using the method of comparison of the observed and expected indexes of the marker, was also increased with the administration of methylcobalamine.

It is known that the pool of proliferating cells increases during the process of the growth of most solid tumors. Ca-755 registered a slow decline (28% to 9%) in the amount of the marked cells on the 6^{th} -14^{th} day after the single administration of ^{3}H-tymidine. The effect of methylcobalamine on Ca-755 also shows the decrease of the marker index, but there is an increased number of the marker cells in the tumor even in the later stages of the proliferation. Thus, the administration of a small dose of methylcobalamine allows to increase the pool of the proliferating cells that are most sensitive to the inhibiting action of the cycle-specific substances. When evaluating the kinetics of the growth and proliferation of the Ca-755 cells, we also looked at the effect of methylcobalamine chloride palladate. Its administration to the animals resulted in a suppression of the tumor growth in the early exponential phase [15]. The index of the proliferating pool in the tumor did not vary from one in the control group 48 hours after the administration of methylcobalamine chloride palladate.

Combining the data that we obtained allowed us to formulate a fundamentally new approach to the combined chemotherapy of tumors. The approach is based on the use of the modifying effect of cobalamines on tumor proliferation. Considering the synergy of the folic acid compounds and cobalamines in the proliferation of cells, it was possible to increase the antitumor effect of methotrexate. We have researched the alternative methods in order to increase the antitumor activity of methotrexate when combined with a cobalamine derivative. An

increase in the selective effect of methotrexate was obtained as a result of the increase in the proliferating pool by means of methylcobalamine. The antitumor effect of methotrexate was significantly increased when combined with methylcobalamine in animals with Ca-755, CC-5 and leucosis L 1210. The antitumor effect of methotrexate – a specific inhibitor of dihydrofolate reductase significantly increases even with the simultaneous blockage of methionine synthase in tumor cells and with the help of an antagonist of methylcobalamine or a derivative of quinoline of Caine, used at the same time [16, 17]. It should be noted that the antitumor activity of the investigated antagonists of methylcobalamine is insignificant and does not provide a long-lasting effect of suppression of the tumor growth. However, limiting the protection pathway of folic coenzymes production creates an additional background that increases the antitumor effect of methotrexate (see the picture). Its combination with methylcobalamine chloride palladate increases the suppression of growth of Ca-755, CC-5, and ACALI and is seen when the activity of methotrexate alone diminishes (table 2).

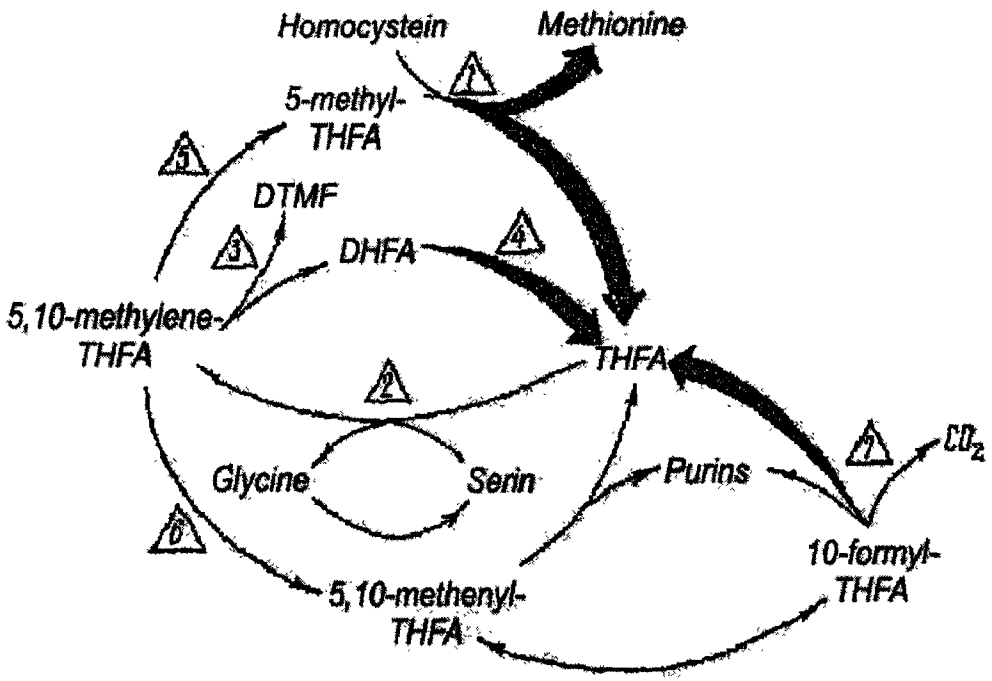
The most important moment in the mechanism of the stimulating action of methylcobalamine is its ability to induce the activity of methionine synthase in tumor cells. It has been demonstrated earlier in the culture of mammal cells and human tumor cells. However, not all types of tumor cells can perform in vitro methionine biosynthesis that is necessary for their growth [18]. That is the reason why our experimental data on the increase in the activity of methionine synthase in Ca-755 cells by the administration of methylcobalamine to animals is very important [15]. It seems that the reduction in the amount of holoenzyme under the effect of methylcobalamine chloride palladate can be explained by a smaller entry of active cobalamine in tumor cells due to the competition for transport protein – transcobe-lamin II. Our data confirms that when the animals were administered methylcobalamine antagonist, the accumulation of cyan-Co^{57}-cobalamine in the exponential stage of the growth in Ca-755 and the production of marked cobalamine enzymes in cells were significantly decreased [19].

The increase in the antitumor activity of methotrexate when used simultaneously with methylcobalamine, as we think, depends mostly on the ability to suppress DNA synthesis in the majority of tumor cell population. Though, the possibility of the effect of methylcobalamine on the speed of methotrexate entry in tumor cells [17] cannot be excluded, it needs additional research. Nowadays we have a new experimental proof of the increase of the antitumor activity of methylcobalamine and other S-phase-specific antimetabolite – arabinosid cytosine with the help of methylcobalamine.

Myasishcheva N.V, Sofina Z.P.

O.D. Golenko, F.G. Arsenyan

Figure on top of page 30, left-hand column



Folic acid cycle and the biosynthesis of methionine, purine and pyrimidines.

1 – cobalamine-dependent methionine synthase, 2- serinoxymethyltransferase, 3 – thymidylate synthase, 4 – dihydrofolatereductase, 5 – methylen-THFA reductase, 6 - methylen-THFA dehydrogenase, 7 - formyl-THFA dehydrogenase.

Table 1 on the bottom of page 30, right-hand column

Table 1, Effect of Methylcobalamine on the growth of subinoculated tumors in mice

Tumor	Dose of the drug	Size of the tumor (% to control)	
		Time after the administration of the drug, days.	
		1-2	7-8
Ca-755	0.01	180	65
ACALI	0.01	126	37
CC-5	0.01	47	0
C-37	0.5	57	0
Notice: Methylcobalamine was administered on 3rd and 7th day of the tumor growth. Results are statistically correct (P less than 0.05) compared with control group.			

Table 2 on the top of page 32, left-hand column

Table 2, Combined effect of methotrexate, cobalamine derivatives and quinoline of Caine on the growth of Ca-755 of mice from C57B1 line.

Drug	Dose of the drug	Suppression of the tumor growth (% to control group)			Increase in animal's life span (% to control group)
		Time after administration of the drug, days			
		1-2	7-8	14-15	
Methotrexate	10	75	44	18	0
Methotrexate and Methylcobalamine	10+0.01	99	70	9*	19*
Methotrexate and Methylcobalamine chloridepalladate	10+250	98	88	---	0
Methotrexate and Methylcobalamine chloridepalladate and Quinoline of Caine	10+250+5	100	96	85	30

Note: The drug was administered on the 3rd day of tumor growth; Methotrexate and quinoline of Caine was administered intraperitoneal twice with the 96 hour interval; Methylcobalamine chloridepalladate was administered 5 times with the 24 hour interval; $p > 0.05$.

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80333 München

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Title:	RU:	Противоопухолевая эффективность метотрексата при его комбинированном применении с кобаламиновыми производными
	EN:	Antitumor activity of methotrexate when used in combination with cobalamine derivatives

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З. П. СОФЬИНА, Н. В. МЯСИЩЕВА, Ф. Г. АРСЕНЯН, А. М. ЮРКЕВИЧ

ВОЗМОЖНОСТЬ УСИЛЕНИЯ ПРОТИВООПУХОЛЕВОГО ДЕЙСТВИЯ АНТАГОНИСТА ФОЛИЕВОЙ КИСЛОТЫ АНАЛОГАМИ МЕТИЛКОБАЛАМИНА

Онкологический научный центр АМН СССР, Москва

Стимулирующее действие цианокобаламина на рост перевиваемых опухолей разного вида животных (саркома кур Рауса, фибросаркома PW-2, саркома 45 и ССК крыс, карцинома Герена, саркома 180 и лимфосаркома мышей) и ослабление лечебного действия некоторых противоопухолевых препаратов при совместном их применении с витамином B_{12}, отмечаемые в ранних исследованиях, обусловлены активным биосинтезом его коферментов в организме животных. Оценка функциональной роли метилкобаламина — одного из кобаламиновых коферментов в процессах роста нормальных и опухолевых клеток — привлекает наибольшее внимание.

Метилкобаламин является коферментом метионинсинтетазной реакции — ключевого звена, определяющего синергизм действия кобаламинов и соединений фолиевой кислоты в процессах клеточной пролиферации. Особая значимость метилкобаламина для активации этой ферментной системы отмечена в результате изучения нарушенного обмена кобаламинов при лейкозах человека. Малая эффективность комбинированной цитостатической терапии при определенных вариантах острого лейкоза, протекающих с высокой концентрацией метилкобаламина в крови, подтверждала специфичность его действия в организме (Н. В. Мясищева и соавт., 1969). В настоящее время установлена активная роль метилкобаламина в процессах пролиферации клеток кроветворной ткани здоровых животных. Под воздействием метилкобаламина в селезенке мышей возрастают число клеток, синтезирующих ДНК, их митотическая активность и величина пролиферативного пула (О. Д. Голенко и соавт.). Обнаружено значительное увеличение частоты развития гемобластозов у мышей при комбинированном введении метилкобаламина с эндогенными бластомогенами. Важным моментом механизма стимулирующего действия кобаламинов является их индуцирующее влияние на активность метионинсинтетазы. В культурах нормальных клеток млекопитающих и опухолевых клеток человека активность метионинсинтетазы заметно возрастает с увеличением содержания кобаламинов в среде культивирования (Mangum и соавт.; Kamely и соавт.). Опухолевые клетки разного типа, однако, отличны от нормальных по своей способности под воздействием кобаламинов усиливать биосинтез метионина, необходимый при интенсивном росте (Halpern и соавт.; Chello и Bertino). Спасательный путь с помощью кобаламинзависимой метионинсинтетазы, обеспечивая увеличение внутриклеточного пула тетрагидрофолиевой кислоты независимо от фолатредуктазной системы, представляет, по-видимому, основной механизм развития устойчивости лейкозных клеток к метотрексату (МТХ) (Н. В. Мясищева; Sauer и Jaenicke).

В связи с этим реальна возможность усиления противоопухолевого эффекта данного метаболита путем его комбинированного применения

с антагонистами кобаламинового кофермента. Понимание механизма действия кобаламинов послужило обоснованием для направленного синтеза аналогов метилкобаламина и их испытания в качестве потенциальных противоопухолевых соединений.

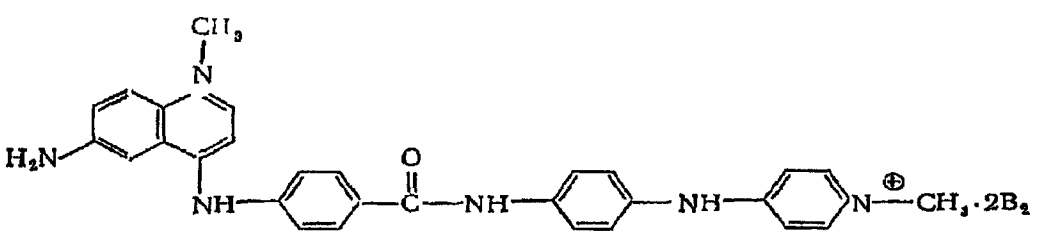
В химиотерапевтических экспериментах были изучены дифторхлорметилкобаламин и хлорпаллодат метилкобаламина, обнаруживавшие активность при исследованиях in vitro в подавлении роста бактериальных клеток и торможении синтеза ДНК в культуре эмбриональных фибробластов человека (Н. В. Мясищева и соавт., 1977).

При разработке схемы комбинированного воздействия были учтены основные аспекты физиологического действия кобаламинов в организме: контроль за поступлением соединений фолиевой кислоты в клетки и образованием коферментов фолата, а также интенсивность поглощения кобаламинов опухолевыми клетками (Burke и соавт.; Tisman и Herbert; Floodh и Ullberg). В связи с этим можно было рассчитывать на избирательность действия исследуемых соединений и возможность снижения активности кобаламинозависимого фермента в организме. Однако трудно было ожидать значительного эффекта при их изолированном применении. Поэтому нам представлялось важным оценить противоопухолевое действие этих соединений на фоне торможения активности дигидрофолатредуктазы с помощью МТХ.

Материал и методы. Исследования проведены на мышах линии $C_{57}BL$, CBA, BALB/c и гибридах $BDF_1/C_{57}BL$ x DBA(2), массой 20—25 г, полученных из питомника АМН СССР. Противоопухолевая активность аналогов метилкобаламина изучена на перевиваемых лейкозах L-1210 и La и солидных опухолях: аденокарциноме молочной железы (Са-755), раке шейки матки (РШМ-5) и аденокарциноме кишечника (АКАТОЛ). Мы выбрали в качестве основного объекта исследования солидные опухоли, на которых легче выявить стимулирующее влияние метилкобаламина, чем на модели лейкозов мышей L-1210 и La, с высоким пролиферативным пулом и весьма короткой продолжительностью жизни животных.

Метилкобаламин (CH_3Cbl) и дифторхлорметилкобаламин (CF_2ClCbl) получены по известному методу (Wood и соавт., 1968), модифицированному в разделе выделения (Е. М. Тачкова и соавт.). Хлорпаллодат метилкобаламина ($MetCbl \cdot PdCl_3$) синтезирован способом Е. Г. Чаусера. Метилкобаламин вводили внутримышечно из расчета 10 мкг/кг 2 раза на курс лечения с интервалом 96 ч, CF_2ClCbl — ежедневно подкожно из расчета 500 мг/кг одномоментно либо 2 раза в день по 250 мг/кг в течение 5 дней. Плохо растворимый хлорпаллодат метилкобаламина вводили перорально в 2% крахмальной суспензии в суточной дозе 500 мг/кг в течение 5 дней или 2 раза с интервалом 96 ч. Суточная доза вводилась одномоментно или по 250 мг 2 раза в день. МТХ фирмы «Lederle» использовали в дозе 10 мг/кг внутрибрюшинно с интервалом 96 ч.

В наших исследованиях активность кобаламиновых производных изучена не только при комбинированном применении с МТХ, но также с хинолиновым производным (NSC-176319):



Препарат получен нами из Национального института рака США в соответствии с соглашением о сотрудничестве между СССР и США в области химиотерапии опухолей. Согласно характеристике, представленной американскими учеными, препарат является ингибитором метионинсинтетазы (Carter и соавт.). Хинолиновое производное применяли внутрибрюшинно в дозе 5 мг/кг ежедневно или с интервалом 96 ч, что составляет половину максимально переносимой дозы для использованного режима. Лечение начинали через 48 ч после перевивки опухоли. Результаты воздействия оценивали через 24 ч после окончания курса лечения и в различные сроки на протяжении жизни животных. Критерием эффективности служили процент торможения роста опухоли, вычисляемой по условному объему, и увеличение продолжительности жизни животных. В каждом из опытов контрольные и опытные группы составляли так, чтобы их численность обеспечивала статистическую значимость минимальных учитываемых процентов торможения

роста опухолей (50%) и увеличения продолжительности жизни мышей (25%). В соответствии с указанными требованиями опытные группы состояли из 6—10 мышей, а контрольные — из 6—13 животных, в зависимости от используемого штамма опухоли.

Результаты и их обсуждение. В проведенных исследованиях впервые обнаружено стимулирующее влияние метилкобаламина на рост перевиваемых опухолей Са-755, АКАТОЛ, в меньшей степени — на рост РШМ-5 (табл. 1). Наибольшая интенсивность роста опухоли под воздействием метилкобаламина наблюдалась при перевивке Са-755 мышам-гибридам BDF_1 (180%) по сравнению с ростом той же опухоли у мышей чистой линии $C_{57}Bl$. Стимуляция размножения опухолевых клеток происходила в период введения метилкобаламина; наибольшее различие в величине опухолей у животных опытной и контрольной групп выявлено непосредственно после окончания введения препарата. В последующие сроки рост опухолей у мышей, получавших метилкобаламин, замедлялся. При перевивке АКАТОЛ мышам разного пола интенсивность роста опухоли при воздействии метилкобаламина различна. Стимулирующее действие препарата было значительнее выражено у самцов (см. табл. 1).

Как и следовало ожидать, изолированное воздействие аналогов метилкобаламинов тормозило рост перевиваемых опухолей Са-755, РШМ-5 в небольшой степени и лишь непосредственно после введения препаратов (табл. 2).

При сравнительной оценке наибольшая ингибирующая активность обнаружена при использовании хлорпаллодоата метилкобаламина. Эффективность торможения роста Са-755 была более выражена у мышей-гибридов BDF_1 по сравнению с мышами $C_{57}Bl$. Как было указано, именно у мышей BDF_1 в значительно большей степени проявлялось и стимулирующее действие метилкобаламина. В этой серии опытов продолжительность жизни мышей BDF_1 с аденокарциномой молочной железы при воздействии CF_2ClCbl и хлорпаллодата метилкобаламина увеличивалась на 50% (см. табл. 2). В то же время при введении производных метилкобаламина отсутствовал эффект торможения роста АКАТОЛ. Отмечено большое различие в действии кобаламиновых производных на опухоль в зависимости от режима их применения (см. табл. 2). По-видимому, при однократном введении большой дозы (500 мг/кг) возможна диссоциация препаратов с последующим образованием активной формы, стимулирующей рост опухоли.

В соответствии с нашим предположением при комбинировании аналогов метилкобаламина с МТХ обнаружено усиление их действия на опухоль (Са-755, РШМ-5; табл. 3). Увеличение противоопухолевого эффекта в результате комбинированного воздействия проявлялось непосредственно после курса введения препаратов и, особенно, в последующий период: когда эффект действия одного МТХ уже отсутствовал, сохранялся достаточно высокий процент торможения роста опухоли.

Таблица 1

Влияние метилкобаламина на рост некоторых перевиваемых опухолей

Опухоль	Доза препарата, мкг/кг	Срок введения препарата после прививки опухоли, дни	Рост опухоли после введения препарата, % к контролю		
			1 день	7 дней	14 дней
Са-755 $C_{57}BL$	10	2-й и 6-й	+74	+21	+23
BDF_1	10	2-й и 6-й	+180	+65	+10
АКАТОЛ:	10	2-й и 6-й	+20	+23	+31
самки					
самцы	10	2-й и 6-й	+126	+37	+33

Примечание. Здесь и в табл. 2—6 знак «плюс» обозначает стимуляцию роста опухоли.

Таблица 2

Противоопухолевое действие аналогов метилкобаламина

Опухоль	Препарат	Доза препаратов, мкг/кг	Срок введения препаратов после прививки опухоли, дни	Торможение роста опухоли, % к контролю			Увеличение продолжительности жизни мышей, % к контролю
				1 день	7 дней	15 дней	
Са-755	Хлордифторметилкобаламин (CF_2ClCbl)	250+250	2—6-й	30	+8		54
РШМ-5		250+250	2—6-й	43	38	0	16
АКАТОЛ		250+250	2—6-й	0	0	0	0
Са-755	Комплекс трихлорметилкобаламина с ($MetCb \cdot PdCl_2$)	250+250	2—6-й	90	59		50
(BDF_4)		500	2—6-й	13	16	20	
РШМ-5		250+250	2—6-й	80	23	0	10
АКАТОЛ		500	2—6-й	+130	+15	+18	0
		250+250	2—6-й	0	0	0	0

Для понимания возможного механизма действия аналогов метилкобаламина в организме животных был осуществлен сравнительный анализ роста тех же опухолевых штаммов при изолированном влиянии ингибитора метионинсинтетазы — хинолинового производного — и его сочетанного воздействия с МТХ. Торможение роста Са-755, РШМ-5 и АКАТОЛ увеличивалось в зависимости от концентрации препарата. Наиболее эффективно препарат воздействовал на Са-755. При увеличении дозы от 5 до 15 мг/кг торможение роста опухоли возрастало соответственно до 40 и 96%. Однако с увеличением дозы препарата заметно возрастала и его токсичность. Например, при штаммах лейкозов L-1210 и La наиболее оптимальной дозой, по нашим данным, являлась доза 10 мг/кг, при которой в 3—4 раза увеличивалась продолжительность жизни животных. При уменьшении дозы эффект воздействия препарата на мышей с лейкозами был существенно ниже. При солидных опухолях в наших исследованиях не было отмечено значительного увеличения продолжительности жизни мышей. При сочетанном введении препарата с МТХ даже в малой дозе (5 мг/кг) наблюдалась суммация эффекта, что подтверждало увеличение торможения роста опухоли (табл. 4). При более позднем начале лечения животных (на 8-й день после перевивки опухоли) и ежедневном введении препаратов в течение 5 сут (5 мг/кг хинолинового производного; 2 мг/кг МТХ) результаты были еще более демонстративны (Са-755), но при суммарном воздействии увеличивалась также и общая токсичность (табл. 5).

Увеличение торможения роста опухоли и продолжительности жизни животных отмечено при комбинированном воздействии хлорпаллодата метилкобаламина и хинолинового производного (NSC-176319; табл. 6). Учитывая усиление действия МТХ при его комбинированном использовании с аналогами метилкобаламина и ингибитором метионинсинтетазы, мы осуществили комбинированное лечение мышей с Са-755 с применением всех 3 ингибиторов: МТХ, хинолинового производного и наиболее активного аналога кобаламинового кофермента — хлорпалладата метилкобаламина (см. табл. 6).

В результате комбинированного применения ингибиторов метионинсинтетазы и дигидрофолатредуктазы значительно усиливалось противоопухолевое действие, особенно в отдаленные сроки после окончания лечения. В этих условиях через 2 нед после окончания введения препаратов торможение роста опухоли составляло 85%, в то время как в группах мышей, получавших каждое из исследуемых соединений изолированно или комбинацию из 2 препаратов, в эти сроки угнетение роста

Таблица 3

Противоопухолевое действие комбинации МТХ и аналогов метилкобаламина

Опухоль	Препарат	Доза введения препаратов, мг/кг	Срок введения препаратов после прививки, дни	Торможение роста опухоли, % к контролю					Увеличение продолжительности жизни мышей, % к контролю
				1 день	5 дней	7 дней	10 дней	14 дней	
Са-755 ($C_{57}BL$)	МТХ	10	2-й и 6-й	75		10	+32		16
	MetCbl·$PdCl_2$	250+250	2-й и 6-й	58		20	14		0
	МТХ+MetCbl·$PdCl_2$	10+250+250 (вводились одновременно)	2—6-й	97		75	0		0
РШМ-5 (СВА)	МТХ	10	2-й и 6-й	90		48		40	0
	MetCbl·$PdCl_2$	500	2-й и 6-й	+220		+100		+80	0
	МТХ+MetCbl·$PdCl_2$	10+500 (вводились одновременно)	2-й и 6-й	97		65		40	40
Са-755 (гибриды)	МТХ	10	2-й и 6-й	87	81	45	67		
	CF_2ClCbl	500	2-й и 6-й	+67	+5	+21	5		
	МТХ+CF_2ClCbl	10+500 (CF_2ClCbl, вводился за 3 ч до МТХ)	2-й и 6-й	97	99	74	67		

Таблица 4

Действие комбинированного применения МТХ и NSC-176319 на опухоли мышей

Опухоль	Препарат	Доза препаратов, мг/кг	Срок введения препарата после прививки опухоли, дни	Торможение роста опухолей, % к контролю				
				1 день	5 дней	7—8 дней	10 дней	14—16 дней
Са-755 (BDF_1)	МТХ	5	2-й и 6-й	46	9	19	19	23
	NSC-176319	5	2-й и 6-й	18	8	41	29	30
	МТХ+NSC-176319	5+5 (вводились одновременно)	2-й и 6-й	81	62	66	43	31
РШМ-5 (СВА)	МТХ	10	2-й и 6-й	69		74		65
	NSC-176319	10	2-й и 6-й	20		55		31
	МТХ+NSC-176319	10+10 (вводились одновременно)	2-й и 6-й	88		84		75
АКАТОЛ (BALB/c)	МТХ	10	2-й и 6-й	45	53	44		
	NSC-176319	5	2-й и 6-й	12	27	30		
	МТХ+NSC-176319	10+5 (МТХ вводился через 20 мин после NSC-176319)	2-й и 6-й	65	43	40		

Таблица 5

Действие MTX и NSC-176319, применяющихся в комплексе, на рост Са-755 мышей

Препарат	Доза препаратов мг/кг	Срок введения препарата после прививки опухоли, дни	Торможение роста опухоли, % к контролю		Отношение числа погибших животных к числу животных в группе
			1 день	3 дня	
MTX	2	8—12	12	+13	1/6
NSC-176319	5	8—12	12	+8	0/6
MTX+NSC-176319	2+5 (вводились одновременно)	8—12	76	79	5/6

Таблица 6

Действие на рост Са-755 мышей MTX, NSC-176319 и комплекса трихлорметилкобаламина с палладием

Препарат	Доза препаратов, мг/кг	Срок введения препаратов после прививки опухоли, дни	Торможение роста опухоли, % к контролю			Увеличение продолжительности жизни, % к контролю
			2 дня	8 дней	14 дней	
MTX	10	2-й и 6-й	99	51	0	14
NSC-176319	5	2-й и 6-й	37	7	+29	0
$MetCbl \cdot PdCl_3$	250	2-й и 6-й	75	40	13	0
NSC-176319+$MetCbl \cdot PdCl_3$	5+250 (вводились одновременно)	2-й и 6-й	90	58	4	23
$MetCbl \cdot PdCl_3$+MTX	250+10 (MTX вводился через 20 мин после $MetCbl \cdot PdCl_3$)	2-й и 6-й	99	58	5	8
NSC-176319+MTX	5+10 (MTX вводился через 20 мин после NSC-176319)	2-й и 6-й	99	88	44	0
NSC-176319+$MetCal \cdot PdCl_3$+MTX	5+250+10 (NSC-176319 и $MetCbl \cdot PdCl_3$ вводились одновременно, а MTX-через 20 мин после них)	2-й и 6-й	00	95	85	20

опухолей практически отсутствовало. Однако следует отметить, что одновременно повышалась токсичность. Установлено также, что действие комбинации препаратов существенно изменялось в зависимости от последовательности введения комбинантов и интервалов между ними. Так, одновременное введение NSC-176319 и МТХ оказалось значительно менее токсичным для организма, чем введение их с интервалом 3 ч при равном противоопухолевом эффекте.

Таким образом, результаты экспериментальных исследований подтверждают наше предположение о возможности усиления противоопухолевого действия МТХ с помощью аналогов метилкобаламина и ингибитора метионинсинтетазы. Это открывает новый подход к лечебному воздействию на опухоли с использованием антагонистов физиологического регулятора обмена соединений фолиевой кислоты в организме. Нами установлена противоопухолевая активность антагонистов кобаламинового кофермента. Однако активность исследованных аналогов метилкобаламина, блокирующих определенные метаболические звенья, недостаточна высока для полного и длительного торможения роста опухоли. Противоопухолевое действие аналогов кобаламинового кофермента может быть значительно усилено путем их комбинированного применения с МТХ. Полученные экспериментальные данные указывают на целесообразность испытания эффективности аналогичного рода комбинаций в клинике. Наша основная задача в настоящее время состоит в разработке оптимального режима комбинированного лечения опухолей указанными препаратами на основе всестороннего анализа механизма их сочетанного действия в организме.

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POSSIBILITY OF POTENTIATING THE ANTINEOPLASTIC ACTION OF FOLIC ACID ANTAGONIST BY METHYLCOBALAMINE ANALOGUES

Z. P. Sofyina, N. V. Myasischeva, F. G. Arsenyan, A. M. Yurkevich

Summary. The effect of methylcobalamine and its analogues (difluoro-chloromethylcobalamine — CF_2ClCbl and methylcobalamine chloropalladate — $MetCbl \cdot DdCl_3$) on the growth of transplantable tumours in mice: adenocarcinoma of the mammary gland (Ca-755), carcinoma of the uterine cervix (CUC-5), carcinoma of the intestine (ACATOL) was studied. The activity of the cobalamine coenzyme analogues was investigated when used alone or combined with inhibitors of dehydrofolate reductase and methyonine synthetase. The results of the experiments indicate a stimulating effect of methylcobalamine on the growth of transplantable solid tumours in the animal organism. The antitumour activity of the methylcobalamine analogues studied was found to be higher in combined application with methotrexate. The most effective inhibition of tumour growth and the longer survival of the animals were achieved in combined application of methylcobalamine with methotrexate and methyonine synthetase inhibitor, depending upon the scheme of administration.

СОДЕРЖАНИЕ

CONTENTS

РЕФЕРАТЫ СТАТЕЙ, ОПУБЛИКОВАННЫХ В ЭТОМ НОМЕРЕ

УДК 61:612.017.1

Иммунология в современной медицине. Косяков П. Н. Вестн. АМН СССР, 1979, № 1, с. 14.

Отмечается значение иммунологии для многих разделов современной медицины: прежде всего иммунологии инфекций, а также многих разделов неинфекционной иммунологии. Иммунологические методы благодаря их уникальной специфичности и высокой чувствительности нашли самое широкое применение в различных областях биологии и медицины. Указывается, что иммунные реакции, защитные по своей природе, в силу тех или других причин могут быть извращены и направлены не только на чужеродные антигены, но и на некоторые собственные, нормальные, неизмененные антигены клеток и тканей, в результате чего возникают истинные аутоиммунные болезни.

Библиография: 15 названий.

УДК 612.017.1:001.8

Современные взгляды на пути развития иммунологии (проблемы и перспективы). Бароян О. В., Каулен Д. Р. Вестн. АМН СССР, 1979, № 1, с. 21.

Представлены основные задачи, стоящие перед иммунологией. Рассматривается главная задача — возможности поисков путей целенаправленной регуляции иммунного ответа организма. Авторы видят такую возможность в разработке способов, регулирующих клеточные кооперации, модификации клеточного микроокружения, использовании фрагментов антител. Обращается внимание на значение растворимых медиаторов клеточного иммунитета — лимфокинов. Особо отмечено влияние

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Sofyina Z.P., Myasisheva N.V., Arsenyan F.G., Yurkevich A.M.

POSSIBILITY TO INCREASE THE ANTITUMOR EFFECT OF FOLIC ACID ANTAGONIST WITH THE HELP OF METHYLCOBALAMINE ANALOGS

The Science Center of Oncology, Academy of Medical Sciences, USSR, Moscow

The stimulating effect of cyancobalamine on the proliferation of subinoculated tumors in different animals (chicken sarcoma of Raus, fibrosarcoma PW-2, sarcoma 45 and CCK of rats, Geren carcinoma, sarcoma 1180 and lymphosarcoma of mice) and the weakening effect of several antitumor medications combined with vitamin B12 that were noted in previous studies can be explained by the active biosynthesis of its enzymes in the body of animals. The evaluation of the functional role of methylcobalamine – one of the cobalamine coenzymes in the proliferation of normal and tumor cells – is of utmost importance.

Methylcobalamine is a coenzyme in the methionine synthase reaction – the key reaction that determines the synergy of cobalamine and folic acid compounds action in cell proliferation. The particular importance of methylcobalamine for the activation of this enzyme complex has been noticed when studying the disturbed metabolism of cobalamines in human leucosis. The low effect of the combined cytostatic therapy in certain types of acute leucosis with the high concentration of methylcobalamine in blood has confirmed the specificity of the latter in a human body (Myasisheva N.V. et al., 1969). The active role of methylcobalamine in the proliferation of the cells of hematopoietic tissue in normal animals has been determined nowadays. Methylcobalamine causes an increase in the number of cells that synthesize DNA in the spleen of mice, its mitotic activity and the size of the proliferating pool (Golenko O.D. et al.). A significant increase of the hemoblastosis in mice after the administration of methylcobalamine with endogenous blastomogenes has been found. The important part of the stimulating action of methylcobalamine is in its inducing effect on methionine synthase. In the cultures of normal cells in mammals and tumor cells in humans the activity of methionine synthase greatly increases with the increase of the concentration of cobalamines in a cultured medium (Mangum et al.; Kamely et al.). However, different types of tumor cells differ from normal cells in their ability to increase methionine biosynthesis required in case of acute proliferation under the influence of

cobalamine (Halpern et al.; Chello and Bertino). Cobalamine-dependent methionine synthase increases the intracellular pool of tetrahydrofolic acid irrespective of the folate reductase system and serves as a main mechanism of resistance of leukemia cells to methotrexate (MTX) (Myasisheva N.V; Sauer and Jaenicke).

Thus, there is a possibility to increase the antitumor effect of a given metabolite by its combination with the antagonists of cobalamine coenzyme. Understanding the mechanism of action of cobalamines allows to explain the directed synthesis of methylcobalamine analogs and investigations of their potential as antitumor compounds.

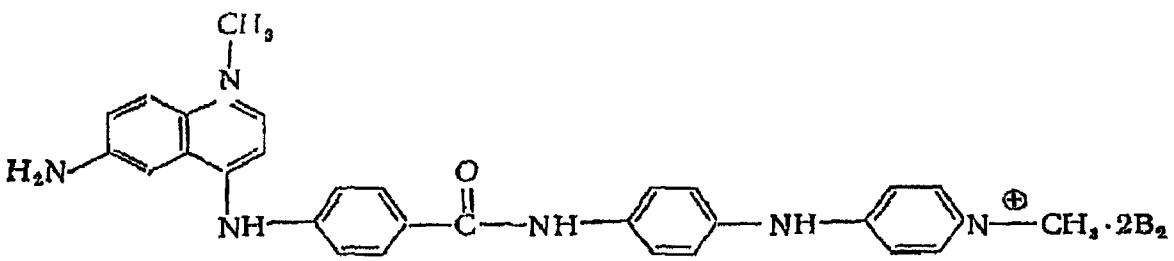
Difluorinechlorinemethylcobalamine and chlorinepalladate methylcobalamine were studied as chemotherapeutic agents. They displayed an in vitro activity when suppressing the proliferation of bacterial cells and DNA synthesis in the culture of embryonic human fibroblasts (Myasisheva N.V. et al, 1977).

While developing the scheme for the combined effect, the main aspects of the physiological action of cobalamines have been taken into account: the control over the entry of folic acid compounds in cells; the production of folate coenzymes and intensity of cobalamine uptake by tumor cells (Burke et al.; Tisman and Herbert, Floodh and Ullberg). It was now possible to count on the selective action of the investigated compounds and their ability to decrease the activity of cobalamine-dependent enzyme in the organism. However, it was difficult to expect a significant effect from their isolated use. Therefore, we found it important to evaluate the antitumor effect of these compounds with the inhibition of dihydrofolatereductase via the administration of MTX. Materials and Methods.

Studies were performed on mice from line $C_{57}BL$, CBA, BACB/c and hybrids $BDF_1/C_{57}Blx$ DBA (2), with weight 20-25 gr from the nursery of the Academy of Medical Sciences of the USSR. The antitumor activity of methylcobalamine analogs was studied on subinoculated leucosis L-1210, La and solid tumors: breast adenocarcinoma (Ca-755), cervical cancer (CC-5) and intestine adenocarcinoma (ACAI). We chose solid tumors to be the main object of our research, because it is easier to determine the stimulating effect of methylcobalamine on solid tumors than on model of leucosis in mice L-1210 and LA with high proliferating pool and rather short life-span of animals.

Methylcobalamine (CH_3Cbl) and difluoridechloridemethylcobalamine (CF_2ClCbl) were synthesized according to the well-known method, altered in the separation phase (Tachkova E.M. et al.). Methylcobalamine chloridepallodate ($MetCbl\text{-}PbCl_3$) was synthesized by the method of Chauser E.G. Methylcobalamine was administered intramuscularly 10 mkg/kg twice during the treatment course with a 96 hour interval, CF_2ClCbl was administered daily subcutaneously 500 mg/kg once a day or twice a day 250 mg/kg for 5 days. Methylcobalamine chloride palladate, which was poorly dissolved, was administered per os in 2% starch suspension once a day 500mg/kg for 5 days, or twice a day with a 96 hour interval.

In our studies, the activity of cobalamine derivatives was determined not only in its combination with MTX, but also with quinoline derivative (NSC-170319).



The drug was delivered from the National Institute of Cancer of the USA according to the agreement about cooperation between USSR and USA in chemotherapy of tumors research. According to the description presented by the American scientists, the drug is an inhibitor of methionine synthase. (Carter et al.) Quinoline derivative was administered intraperitoneally 5 mg/kg daily or with a 96 hour interval, which is half of the safety dose for the chosen regime. The treatment began 48 hours after the tumor subinoculation. The effect was evaluated 24 hours after the end of the treatment and at different times throughout the animals' lives. The percentage of the tumor proliferation suppression was chosen to be the criterion for effectiveness measured based on the specific volume and increase of duration in life of animals. In each experiment the control and experimental groups were composed in such a way that its numbers would assure a statistic significance at a minimal percentage of the tumor proliferation (50%) and an increase of the life-span in mice (25%). According to the mentioned criteria, the experimental groups contained 6-10 mice, and control groups contained 6-13 mice, depending on the tumor.

Results and Discussion

Our studies revealed a stimulating effect of methylcobalamine on the proliferation of subinoculated tumors Ca-755, ACAI, and to a smaller degree on the proliferation of CC-5 (table 1). The main intensity of the tumor proliferation under the influence of methylcobalamine was seen in mice-hybrids BDF_1 (180%) that were subinoculated with Ca-755 than that in mice of the clean line $C_{57}Bl$. The stimulation of the proliferation of the tumor cells occurred during the administration of cobalamine; the main difference between the sizes of the tumors in animals of the experimental and control groups was determined immediately after the administration of the drug. Later the tumor growth in mice receiving methylcobalamine slowed down. In mice inoculated with ACAI the intensity of the tumor proliferation under the influence of methylcobalamine varied. The stimulating effect of the drug was more significant in male mice (table1).

As it was expected, an isolated effect of methylcobalamine analogs decreased the proliferation of subinoculated tumors Ca-755, CC-5 to some degree and only immediately after the administration of the drugs (table 2).

In comparison, the inhibiting activity was the highest with methylcobalamine chloride palladate. The effective suppression of the Ca-755 proliferation was more significant in mice-hybrids BDF_1 compared to mice $C_{57}Bl$. As it was already mentioned, the stimulating effect of methylcobalamine was also the most significant in mice-hybrids BDF_1. In this series of experiments the life-span of the BDF_1 mice with breast adenocarcinoma had a 50% increase under the effect of CF_2ClCbl and methylcobalamine chloridepallodate (table 2). At the same time there was no effect of the proliferation in mice with ACAI. The regime of administration of the drug showed a significant difference in the effect of cobalamine derivatives (table 2). Thus, a single administration of a large dose (500 mg/kg) creates the dissociation of the drugs with the following formation of the active form that stimulates the growth of the tumor.

According to our assumption, the combination of analogs of methylcobalamine and MTX shows an increase in its effect on the tumor (Ca-755, CC-5; table 3). The increase of the antitumor effect as a result of the combination of the drugs was seen immediately after the administration of the drugs and, especially, in the following period: when the effect of MTX decreased, there

was still a high percentage of suppression of the tumor growth.

Table 1

Effect of methylcobalamine on the growth of some of the inoculated tumors

Tumor	Administered dose of the drug mg/kg	Time of drug administration after the subinoculation of tumor, days	Growth of the tumor after the administration of the drug, % compared to control group		
			1 day	7 days	14 days
Ca-744, $C_{57}BL$	10	2nd and 6th	+74	+21	+23
BDF_1	10	2nd and 6th	+180	+65	+10
ACAI	10	2nd and 6th	+20	+23	+31
male					
female	10	2nd and 6th	+126	+37	+33
Note: Here and in tables 2-5, + symbolizes stimulation of the tumor growth					

Table 2

Antitumor effect of the methylcobalamine analogs

Tumor	Drug	Administered dose	Time of administration of the drug after subinoculation of the tumor, days	Reduce of tumor growth,% compared to control			Increase of mice life span, % compared to control
				1^{st} day	2^{nd} day	3^{rd} day	
Ca-755	Chloridedifluoride-	250+250	2^{nd}-6^{th}	30	+8		54
	methylcobalamine	250+250	2^{nd}-6^{th}	43	38	0	16
CC-5	(CF_2ClCbl)	250+250	2^{nd}-6^{th}	0	0	0	0
ACAI							
Ca-755	Trichloride-	250+250	2^{nd}-6^{th}	90	59		50
(BDF_1)	methylcobalamine	500	2^{nd}-6^{th}	13	16	20	
	with	250+250	2^{nd}-6^{th}	80	23	0	10
CC-5	($MeCbl*PdCl_3$)	500	2^{nd}-6^{th}	+130	+15	+18	0
ACAI		250+250	2^{nd}-6^{th}	0	0	0	0

In order to understand a possible mechanism of the action of methylcobalamine analogs in the organism of animals we performed a comparative analysis of the same tumor growth under the effect of the isolated influence of methionine synthase inhibitor – quinoline derivative - and its combined effect with MTX. The suppression of the growth of Ca-755, CC-5 and ACAI increased depending on the concentration of the drug. The effect of the drug on Ca-755 was most noticeable. By increasing the dose from 5 to 15 mg/kg, the tumor growth decreased to 40% and 96% correspondingly. However, an increase in the dose of the drug led to the increase in its toxicity. For example, the optimal dose for leucosis L-1210 and La was, according to our data, 10 mg/kg and it is only allowed to increase 3-4 times in a life span. A decrease in the dose led to the decrease in its effect. The solid tumors did not show any difference in the life span of mice. The

combination of the drug with MTX even in a small dose (5 mg/kg) revealed a combined effect that was confirmed by the reduction of the tumor growth (table 4). A later start of the treatment (8^{th} day after the subinoculation of the tumor) and daily administration of drugs for 5 days (5 mg/kg of quionoline derivative; 2 mg/kg MTX) revealed more significant results (Ca-755), but the combination therapy led to the increase in general toxicity (table 5).

The increase in the reduction of the tumor growth and life span of the animals was noticed when using the combination of methylcobalamine chloride palladate and quinoline derivative (NSCp176319, table 6). Considering the increase of MTX effect in case of its combination with methylcobalamine analogs and with inhibitor of methionine synthase we performed a combined treatment of mice with Ca-755 and all 3 inhibitors: MTX, quinoline derivative and the most active analog of cobalamine coenzyme – methylcobalamine chloride palladate (table 6).

The combination of the inhibitors of methionine synthase and dihydrofolatereductase showed a significant increase in the antitumor effect, especially later after the termination of the treatment. Under these conditions the reduction of the tumor growth was 85% 2 weeks after the drug administration was stopped. At the same time, there was almost no inhibition of growth in the groups of mice that were administered just one drug or a combination of two drugs at a time. It should be noted that the level of toxicity has increased with the increase of the effectiveness. We also found out that the effect of combining the drugs for administration was different and depended on the sequence of the drugs and intervals between their administration. Thus, the simultaneous administration of NSC-176319 and MTX appeared to be less toxic than the administration of the same drugs with a 3 hour break. It, however, brought the same antitumor effect.

Having said that, the results of our experimental research supports our thesis about a possible increase of the antitumor effect of MTX with the use of methylcobalamine analogs and methionine synthase inhibitor. The obtained results open a new approach to the treatment of tumors with the antagonists of the physiological regulator of folic acid metabolism compounds in the organism. We have established the antitumor activity of the antagonists of cobalamine coenzyme. However, the activity of known methylcobalamine analogs that block some metabolic pathways is not strong enough for a complete and longer reduction of the tumor growth. The antitumor effect of analogs of cobalamine coenzyme can be increased by means of its combination with MTX. The available experimental data shows necessity of investigation of the

effect of such combinations in clinic. Our main purpose nowadays is to develop an optimal regime for the combined treatment for tumors with the previously mentioned drugs, based on the thorough analysis of the mechanism of its combined effects in the organism.

Table 3 (on top of page 76)

Antitumor effect of MTX and analogs of methylcobalamine

Tumor	Drug	Dose of drug administered, mg/kg	Time of drug administration after the inoculation of tumor, days	Suppression of the tumor growth (% to control group)					Increase of life-span (% to control group)
				1 day	5 days	7 days	10 days	14 days	
Ca-755	MTX	10	2^{nd} and 6^{th}	75		10	+32		16
(C_{57}BL)	MetCbl, $PbCl_3$	250+250	2^{nd} and 6^{th}	58		20	14		0
	MTX + MetCbl	10+250+250	2^{nd}-6^{th}	97		75	0		0
	$PdCl_3$	(simultaneously)							
CC-5	MTX	10	2^{nd} and 6^{th}	90		48		40	0
(CBA)	MetCbl*$PdCl_3$	500	2^{nd} and 6^{th}	+220		+100		+80	0
	MTX + MetCbl	10+500	2^{nd} and 6^{th}	97		65		40	40
	$PdCl_3$	(simultaneously)							
Ca-755	MTX	10	2^{nd} and 6^{th}	87	81	45	67		
(hybrid)	CF3ClCbl	500	2^{nd} and 6^{th}	+67	+5	+21	5		
	MTX + CF_2ClCbl	10+500, CF_2ClCbl was administered 3 hours prior to MTX	2^{nd} and 6^{th}	97	99	74	67		

Table 4 (on the bottom of page 76)

Effect of Methotrexate and NSC 176319 on the tumor in mice

Tumor	Drug	Dose of the drug administered	Time of drug administration after tumor inoculation, days	Suppression of the tumor growth, % to control group				
				1 day	5 days	7-8 days	10 days	14-16 days
CA-755	MTX	5	2nd and 6th	46	9	19	19	23
(BDF_1)	NSC-176319	5	2nd and 6th	18	8	41	29	30
	MTX+NSC-176319	5+5	2nd and 6th	81	62	66	43	31
		(simultaneously)						
CC-5 (CBA)	MTX	10	2nd and 6th	69		74		65
	NSC-176319	10	2nd and 6th	20		55		31
	MTX+NSC-176319	10+10	2nd and 6th	88		84		75
		(simultaneously)						
	MTX	10	2nd and 6th	45	53	44		
ACALI	NSC-176319	5	2nd and 6th	12	27	30		
(BALB/c)	MTX+NSC-176319	10+5 (MTX administered 20 min after NSC-176319)	2nd and 6th	65	43	40		

Table 5 (on top of page 77)

Effect of combination of MTX and NSC 716319 on CA-755 in mice

Drug	Dose of the Drug	Time of administration of the drug after the inoculation of the tumor, days	Suppression of the tumor growth, % to the control group		Ratio of dead animals to the number of animals in the group (%)
			1 day	3 days	
MTX	2	8-12	12	+13	1/6
NSC-716319	5	8-12	12	+8	0/6
MTX+NSC-176319	2+5 simultaneously	8-12	76	79	5/6

Table 6 (on the bottom of page 77)

Effect of MTX, NSC716319 and trichloridemethylcobalamine with palladium on mice CA-755

Drug	Dose of the drug	Time of administration of the drug after the subinoculation of the tumor, days	Suppression of the tumor growth, % to control group			Increase in animals life span, % to control group
			2 days	6 days	14 days	
MTX	10	2^{nd} and 6^{th}	99	51	0	14
NSC-716319	5	2^{nd} and 6^{th}	37	7	+29	0
MetCbl*$PbCl_3$	250	2^{nd} and 6^{th}	75	40	13	0
NSC-716319+MetCbl*$PdCl_3$	5+250 simultaneously	2^{nd} and 6^{th}	90	58	4	23
MetCbl*$PbCl_3$+MTX	250+10 (MTX administered within 20 min after MetCbl*$PdCl_3$	2^{nd} and 6^{th}	99	58	5	8
NSC-716319+MTX	5+10 (MTX administered within 20 min after NSC-716319)	2^{nd} and 6^{th}	99	88	44	0
NSC-176319+	5+250+10	2^{nd} and 6^{th}	00	95	85	20
MetCbl*$PbCl_3$+MTX	(NSC-176319 and MetCbl*$PbCl_3$ simultaneously but MTX within 20 minutes after the others		1			

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EN: POSSIBILITY TO INCREASE THE ANTITUMOR EFFECT OF FOLIC ACID ANTAGONIST WITH THE HELP OF METHYLCOBALAMINE ANALOGS

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LINGO Language Services GmbH
Westenhellweg 85-89
44137 Dortmund/Germany
Amtsgericht Dortmund HRB 21358
Geschäftsführer Eric LINGO

Tel. +49 (0)231 555 799-00
Fax +49 (0)231 555 799-11
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19. Antimetabolites – Preclinical and clinical studies (part 2)

289 LY231514 and its polyglutamates exhibit potent inhibition against both human dihydrofolate reductase (DHFR) and thymidylate synthase (TS): multiple folate enzymes inhibition

C. Shih[1], L. Gossett[1], S. Gates[1], W. MacKellar[1], L. Mendelsohn[1], D. Soose[1], V. Patel[1], W. Kohler[2], M. Ratnam[2]. [1]Lilly Research Lab., Indianapolis, IN and [2]Medical College of Ohio, Toledo, OH, U.S.A.

LY231514, a novel multitargeted antifolate, is currently undergoing extensive multicenter phase II clinical trials. Previous studies have demonstrated that LY231514 inhibited the enzyme thymidylate synthase (TS) derived from mouse lymphoma (Ki = 440 nM, Taylor et al., J. Med. Chem., 35, 4450, 1992). LY231514, one of the best known substrates for the enzyme folylpolyglutamate synthetase (FPGS), is extensively metabolized to the corresponding polyglutamates once it enters the cells. The polyglutamate of LY231514(Glu5) was found to have significant enhanced inhibitory activity against the mouse TS (Ki = 3.4 nM) when compared with the parent monoglutamate. Promising antitumor responses have recently been observed in phase I trials of LY231514 (Rinaldi et al., Proceedings of ASCO, 14, 474, 1995). It was intriguing to find that some patients who had failed on other TS specific agents such as ZD1694 (paltitrexid) and 5-FU/Leucovorin, responded to LY231514 treatments. This clinical observation along with our earlier finding that thymidine alone was not able to fully reverse the cytotoxic effect of LY231514 in culture (Schultz et al., NCI-EORTC, 1996), prompted us to investigate in more detail the modes of action of this novel antifolate antimetabolite. We now report that the polyglutamates of LY231514 not only are potent inhibitors (Ki ~20 nM) of human recombinant TS (rTS), but are also very tight-binding inhibitors of human recombinant dihydrofolate reductase (rDHFR). LY231514 pentaglutamate(Glu5) had a Ki of 100 pM against human DHFR. The parent monoglutamate LY231514 was also found to exhibit tight-binding inhibition against human DHFR (Ki = 300 pM). These results suggest that LY231514 acts upon multiple intracellular targets and that the antitumor effect of this novel antifolate may be derived from its simultaneous inhibition of multiple folate-requiring enzymes. A detailed study that compares the enzyme inhibition of LY231514, ZD1694 and their polyglutamates against human rDHFR and rTS will be presented.

290 Comparative antitumor activity of the multitargeted antifolate LY231514 and the thymidylate synthase (TS) inhibitor ZD1694

R. Schultz[1], S. Andis[1], V. Chen[1], L. Mendelsohn[1], V. Patel[1], C. Shih[1], J. Houghton[2]. [1]Lilly Research Laboratories, Indianapolis, IN 46285; [2]St. Jude Children's Res. Hospital, Memphis, TN 38101

LY231514 and ZD1694 (paltitrexid) are folate analog antitumor agents whose primary mode of action has been ascribed to inhibition of TS. Therefore, salvage of exogenous thymidine (dThd) should circumvent the cytotoxicity of these agents. With ZD1694, we found that the addition of 5 μM dThd fully protected CCRF-CEM leukemia (IC_{50} increased from 8 nM to >40 μM) and GC3/C1 colon carcinoma cells (IC_{50} increased from 4 nM to >40 μM). In contrast, dThd at 5 μM only increased the IC_{50} of LY231514 vs CCRF-CEM cells from 25 to 138 nM (5.5-fold) and GC3/C1 cells from 34 to 837 nM (18.7-fold). Hypoxanthine (100 μM) alone did not influence the cytotoxicity of LY231514. However, the combination of dThd plus hypoxanthine totally protected these cells (IC_{50}s >40 μM). These findings along with recent enzyme studies (Shih et al., NCI-EORTC, 1996) suggest that inhibition of dihydrofolate reductase or other enzymes along the purine de novo pathway may be an important secondary site of action for LY231514. In contrast, the cytotoxicity studies in CCRF-CEM and GC3/C1 cells suggest that TS is the sole target for ZD1694. In addition, we observed that dThd at physiologic mouse plasma levels (1 μM) did not significantly alter the cytotoxicity of LY231514 for GC3/C1, but markedly protected the cells from ZD1694 (IC_{50} increased from 8 nM to 513 nM). Thymidine did not modulate the cytotoxicity of LY231514 and ZD1694 in GC3/TK-, a thymidine kinase-deficient line derived from GC3/C1. Studies with mutant cell lines demonstrated that LY231514 and ZD1694 require polyglutamation and transport via the reduced-folate carrier for cytotoxic potency.

291 Clinical phase I study of LY231514, a multitargeted antifolate, administered by daily x 5 q 21 Schedule

A.C. McDonald, P.A. Vasey, J. Walling[a], MJ Lindb, NP Bailey[b], N Siddiquib, C. Twelves, J. Cassidy & S.B. Kaye. CRC Dept of Medical Oncology, Western Infirmary, Glasgow, [a]Lilly Industries & [b]Division of Oncology, Newcastle General Hospital, Newcastle, U.K.

LY231514 is a quinazoline inhibitor of various enzymes in the folate pathway including thymidylate synthase, dihydrofolate reductase and C1THF reductase. LY231514 has pre-clinical activity in murine tumours and human colonic xenograft models.

This study aims to determine the MTD and toxicity of LY231514, administered by intravenous infusion over ten minutes, daily for five days, repeated every three weeks. To date, 33 patients (mean age 58yrs; 18F, 15M; median PS 1, range 0–2) representing 10 solid tumour types (colo-rectal 17, pancreatic 4, melanoma 2, NSCLC 2, others 8) refractory or not amenable to standard therapy have been treated at 9 dose levels (0.2–4.0 mg/m²) for a total of 99 courses, ranging from 1 to 10 (median 2) courses per patient. Previous chemotherapy had been administered to 28 and radiotherapy to 11 patients. Five had received no previous anti-neoplastic therapy.

Thirty two patients were evaluable for toxicity. (One patient did not complete first course of treatment due to progressive disease). Two principle toxicities have been found, myelosuppression and transaminase elevation. Significant haematologic toxicity (>CTC grade II) was not seen in patients receiving less than 3 mg/m². One patient treated at this dosage developed uncomplicated, reversible grade III neutropenia; a further patient treated at 4 mg/m² demonstrated similar myelosuppression. Significant thrombocytopenia has not been evident. Reversible, mild (CTC grades I–II) derangement of hepatic enzymes was seen at most dose levels. One patient at 2.3 mg/m² had CTC grade III hepatic toxicity. Mild (CTC grades I–II) fatigue occurred in 7 patients, unrelated to either dose or hepatotoxicity. Nausea and vomiting was inconsistent and mild (CTC grades I–II).

One patient with NSCLC, treated at 3 mg/m² has clinical and radiological signs of disease response. The study continues to accrue patients at 4 mg/m² in order to define the MTD.

292 Phase I and pharmacokinetic study of 3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)-quinazoline dihydrochloride (THYMITAQ®, AG337)

P.J. Creaven[1], L. Pendyala[1], N.J. Meropol[1], E.Y. Wu[2], N.J. Clendeninn[2]. [1]Roswell Park Cancer Institute, Buffalo, NY 14263; [2]Agouron Pharmaceuticals, Inc., San Diego, CA 92121 U.S.A.

AG337 is a non-classical antifolate synthesized to fit and bind to the folate cofactor binding site of the enzyme thymidylate synthase. K_i for the enzyme is 1.1×10^{-9} M. AG337 is active against a number of preclinical tumor models. In a phase I study of 5 day continuous infusion (C.I.), the dose limiting toxicity was myelosuppression and mucositis: maximum tolerated dose (MTD) was 1130 mg/m²/day (5.65 g/m²/course) (Rali, I. et al., Proc AACR 36: 240, 1995). Because of preclinical data indicating that more prolonged cellular exposure might be advantageous, a phase I/pharmacokinetic study of 10 day C.I. of AG337 given by portable pump to ambulatory patients (pts) with advanced solid tumors who have failed conventional therapy has been initiated. Doses (mg/m²/d) of 360 x 7, 360 x 10, and 720 x 10 have been studied. The current dose is 900 mg/m²/d x 10. Toxicities on the first 2 courses at doses up to 720 mg/m²/d x 10 have been mild (maximum grade II). Grade II toxicities have included leucopenia, mucositis, fatigue, skin rash and constipation. The MTD has not been reached.

Plasma levels have been evaluated in patients at steady state and post-infusion by a validated, reverse phase, isocratic HPLC method with UV detection at 273 nm. In the first 5 pts studied (2 at 360 x 7, 2 at 360 x 10 and 1 at 720 x 10), C_{ss} ranged from 1.9 to 3.6 μg.ml⁻¹, mean $t_{1/2}$ was 2.5 ± 0.9 h, plasma clearance was 9.3–21.3 L.h⁻¹ and AUC was 336–720 μg.ml⁻¹.h.

A ten day C.I. of AG337 is well tolerated up to doses of 720 mg/m²/d, (7.2 g/m²/course), a larger dose/course than that tolerated with a 5d C.I. Preliminary pharmacokinetics indicate wide interpatient variability but little intrapatient variability in C_{ss}. The study is ongoing, with completion anticipated by February 1996.

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DENNIS S. FERNANDEZ
Fernandez & Associates L.L.P.
1047 El Camino Real, Suite 201
Menlo Park, CA 94025
dennis@iploft.com
www.iploft.com

JAMES T. HUIE
Fernandez & Associates L.L.P.
1047 El Camino Real, Suite 201
Menlo Park, CA 94025
jhuie@scu.edu

ABSTRACT

The patentability of products is essential in the biotechnology field, for limited market exclusivity compensates biotech companies' investments in research and development. The biotechnology field also uniquely faces Federal Drug Administration (FDA) approval, which includes considerable additional expense and time issues a biotech company must address. Although balancing the patent and FDA approval processes may be complex, various strategies of patent extension, of accelerating approval processes, and of prolonging generic drug companies' market entry can yield higher profit returns and maximize value company value.

Key Words – United States Patent and Trademark Office (USPTO), Federal Drug Administration (FDA), biopharmaceuticals, FDA clinical studies, New Drug Application (NDA), market exclusivity, generic market entry, Abbreviated New Drug Application (ANDA), patent term extensions, accelerated approval process.

I. INTRODUCTION

Biotechnology startups and their investors are primarily concerned with optimizing the value of the company. A company's value can be measured by the quality and lifetime of its patents. Longer patent terms produce longer market exclusivity, which consequentially leads to increased profits and value. Patents are crucial to protect a company's ideas while FDA approval is necessary to legally market their products. This article addresses and outlines strategies to extend patent terms and maximize market exclusivity while addressing FDA timing considerations.

II. OVERVIEW OF PATENT AND FDA APPROVAL PERIODS

2.1 Patent Approval process

The average prosecution time for a US patent is 3.4 years while the average biotech patent is 4.4 years. Patents require novelty, utility, and unobviousness. If the patent is granted by the United States Patent and Trademark Office (USPTO), then a 20-year monopoly is granted to the inventor in exchange for public disclosure of the invention.

2.2 Preclinical Studies

Preclinical studies offer predictions and provide safety data for initial studies in humans. Researchers use *in vitro* studies and animals with analogous genetic structure, pharmacodynamic responses, metabolic profiles, cellular receptor interactions, and general physiology to humans. Preclinical studies vary on a case by case basis, depending on the complexity and success of initial research.

2.3 Federal Drug Administration Approval Process

Federal Drug Administration (FDA) approval usually requires 10 to 12 years of development and 100 – 500 million dollars in development costs. The FDA approval period is split between the clinical trials and New Drug Application (NDA) approval. During the clinical trials, the FDA uses test populations to study safety, dosage, pharmacologic and metabolic effects, potential side effects, and effectiveness of the product. The NDA process then comprehensively analyzes the

preclinical and clinical reports, applying a risk-benefit analysis to determine if the product will benefit the public at large.

III. PROPER TIMING OF USPTO AND FDA FILINGS TO MAXIMIZE MARKET EXCLUSIVITY

Large expenses accumulate throughout research, development, and FDA approval of a particular biotech product. A longer patent term provides extended market exclusivity, which allows a company to recover its expenses and produce profits. Every day of market exclusivity is a potential profit for a pioneering company because generic drug companies capture 57.6% market share upon entering the market. Therefore expedient and efficient USPTO and FDA approval is necessary to maximize company profits.
See Figure 1

3.1 Beginning with Preclinical Studies

After the initial idea, preclinical studies should be the first step in the USPTO/FDA processes. Biotech patents regularly require experimental evidence to satisfy the utility requirement. Although researchers can concurrently conduct preclinical studies during patent approval process, basic *in vitro* and animal testing effectively support the patent claims. Regarding the FDA, preclinical studies are the rate limiting step for later FDA clinical development because clinical trials cannot begin until there are sufficient extrapolation predictions for human testing. Therefore, preclinical studies should be preformed as soon as possible to expedite the FDA and USPTO processes.

3.2 Filing Patent with USPTO

The largest obstacle for patent applications is the utility requirement. Occasionally an application's utility may not be clear enough without FDA approval. Therefore it is good practice to emphasize practical functionality in the application, along with substantial preclinical evidence.

Nevertheless patent approval strategically should come before FDA trials in view of certain considerations. If the innovating company begins FDA process before USPTO filing, then it runs the risk of another company patenting the invention before them. Consequently the innovating company would have to license the biopharmaceutical, losing royalties, market exclusivity, and company value; or would have to abandon the FDA process and forfeit millions spent in research and development. Even if the another company does not patent the biopharmaceutical, the innovating company must be careful not to disclose the invention, otherwise it has one year to file the patent before it becomes property of the public domain (internationally, the patent application must be filed before disclosure). Furthermore, issued patents drive FDA approval, speeding up the process. Finally, filing patent applications and receiving approved patents will attract investors that will provide the necessary capital to fund the costly FDA clinical trials.

3.3 Publication of Innovation

In addition to *in* vitro and animal data, safety measures, and predicted dosage, the FDA requires demonstration through review of scientific literature before FDA clinical trials can begin. As mentioned above, the required publication by the FDA should be disclosed *after* the patent has been filed, or the company runs the risk of missing the one-year deadline for patentability.

3.4 Initiating the FDA Approval Process after the Patent Issues and after Preclinical Studies

It is advantageous to immediately begin FDA clinical trials immediately after patent prosecution with the USPTO and preclinical studies have commenced. However a complex issue is to accurately time preclinical studies to end before or concurrently with patent issuance. Each day preclinical studies extend past the issuance date, FDA approval is potentially delayed and the innovating company loses opportunity to exercise market exclusivity.

3.5 Asserting Market Exclusivity after FDA Approval

Once the FDA has approved the biopharmaceutical for US consumers, the

innovating company enjoys market exclusivity for the rest of its patent term. Strategically written patents will effectively and efficiently protect against product infringement by other companies. Including capturing exclusive profits from their product, the innovating company should build reliance on its products to secure its market share once the patent term ends.

IV. EXTENDING THE PATENT TERM AND MARKET EXCLUSIVITY AFTER THE PATENT TERM ENDS

Once the patent term ends, the innovating company loses its market exclusivity privilege as generic manufactures enter the market. There however are processes to extend the life of a patent term through "patent term restoration." Additionally, the innovating company still enjoys market exclusivity while generic manufactures undergo their required FDA approval process. Finally, there are strategic defenses delay generic market entry. The methods to increase market exclusivity are crucial to maximizing overall profits.

4.1 Patent Term Restoration

The USPTO grants patent extensions to compensate for delays in USPTO examinations and prosecution that extend past three years. Thus the average 1.4 years past the three year mark during prosecution may be tacked onto the 20 year patent term.

Another method of patent extension, due to the FDA approval process, is under the Drug Price Competition and Patent Term Restoration Act of 1984, also known as the Hatch-Watchman Act. The act provides a maximum 5-year extension, and is limited to a 14-year term from the time of FDA approval. The calculation of extension is complex and depends on patent prosecution and approval factors.

4.2 Blocking Generic Manufacturers' ANDA's

After the innovating company's patent term expires, generic companies can begin their FDA approval process on their generic drug equivalent. While the innovating company's FDA approval took 10 – 12 years, the Hatch-Waxman Act allows generic companies to use the Abbreviated New Drug Approval (ANDA) process to gain approval within six months.

The requirements for a generic company to file an ANDA application are they must 1) show that the proposed generic drug is the same as, or bioequivalent to, an FDA approved drug; 2) certify that the approved drug was protected by a patent; and 3) the applicant does not use a method of producing the proposed generic drug that is protected by a "method of production" patent.

Because a "production method" patent can be separate from a "drug composition" patent, a tactful patent strategy is to file the production method patent a few years after filing the composition patent. Therefore although the composition would be public domain, the production method's term would still be running and thus be protected. Put simply, a generic has access to the product itself, but does not have rights to produce the product according to the patented method. This strategy is even more effective with biopharmaceuticals than with traditional chemical pharmaceuticals because of the complexity of macromolecules. While there may be more than one method to synthesize a chemical compound, allowing competitors to design around the method of production patent, it is difficult to engineer around complex microbiological systems. Thus, a delayed production method patent can extend market exclusivity of a biopharmaceutical by protecting its production.*

4.3 Delay Through the "Metabolite Defense"

The "metabolite defense" can be used to stall generic market entry. Metabolites are the metabolized derivatives of the original structure, formed after being introduced into and processed by the body. The strategy is to file patents for the metabolites in years subsequent to the filing date of the main patent. Once the generic version is marketed, the innovating company holding the metabolite patent can bring a patent infringement claim against generic company because the generic company will be making products that inevitably become infringing products once digested by consumers. While the metabolite defense has never actually prevailed in court, the

litigated dispute can delay the generics' market entry for up to six months. This extended market exclusivity leads to increased profits by the innovating company.

4.4 Delay Through Raising "Citizen Petitions"

Similar to raising the metabolite defense in court, an innovating company can file a "citizen petition" with the FDA, which raises safety objections with the particular biopharmaceutical. Although the majority of petitions are rejected by the FDA or withdrawn by companies, the petition delays the FDA review staff from and generic market entry for 6 months or more.

V. AVENUES TO ACCELERATE THE INNOVATING COMPANY'S MARKET ENTRY THROUGH USPTO AND FDA EXCEPTIONS

5.1 USPTO Petition to "Make Special"

One procedure to shorten the USPTO process is to make the application "special," in which the USPTO examiner will process the special patent application before all other categories of applications. The USPTO provides special provisions for biotech inventions that allow a biotech patent to have "special" status. To qualify for a petition to make special, the company must be a "small entity," which is a company with fewer than 501 employees or a nonprofit organization. The petition must also state that the patent applicant's technology will be significantly impaired if a patent examination is delayed. If the situation calls for special status, the FDA approval process can be started earlier and can result in extended market exclusivity.

5.2 FDA's "Well Characterized" Biological/Biotech Products

The FDA can assign a biopharmaceutical as a "well characterized" biotech product if its identity, purity, potency, and quality can be substantially determined and controlled. This status allows a company to alter its manufacturing technologies as long as it can produce the same product. In the past, a company had to establish a fully developed process for the product before clinical trials could begin, and if it wanted to change its process it would have to repeat clinical trials again. However with a well characterized biotech product, a company can immediately begin FDA clinical trials once it has the product and improve the manufacturing process at a later date.

5.3 FDA's "Expanded Access" Exception

Using Treatment-IND and "compassionate use" single-patient protocols, companies can market unapproved therapies that are undergoing clinical trials when no satisfactory alternatives are available. If the product is appropriate for the healthcare environment, marketing products concurrently with FDA clinical trials can significantly increase profits.

5.4 FDA's "Accelerated Approval" Process

The "accelerated approval" process allows marketing products to patients with serious or life-threatening conditions. A biopharmaceutical's approval may be accelerated if there are adequate and well-controlled clinical trials that ascertain the biopharmaceutical's clinical outcome will provide a considerable therapeutic benefit over existing therapies.

VI. UNIQUE EXAMPLES OF HOW PHARMAGENOMIC INVENTIONS RELATE TO USPTO AND FDA TIMELINES

6.1 Systems Biology

Systems biology currently is in the initial stages of biotechnology converging with information technology software. The systems biology field primarily deals with programmable software for analyzing biological interactions and structures. Because the software processing does not directly affect the human body, system biology inventions would not have to go through the FDA approval process. It would however have to go through the standard patent approval process.

6.2 Biosensors

As a concept, biosensors can be broadly defined as a sensor to detect biological activity at either molecular or macroscopic levels. As technology

advances, biosensors are being used in microarrays to monitor hybridization or can be implanted *in vivo*. FDA examination is only necessary if the biosensor it will directly affect a human system. If a biosensor is used for *in vitro* research, it will not have to undergo FDA approval.

6.3 Future Integration of Bioinformatics into FDA Trials

In the near future, bioinformatics will efficiently speed up FDA clinical trials. Industry reports predict cutting out about 4 years from the FDA approval process. Establishing an FDA bioinformatics infrastructure will potentially lead to many subtle implications, such as how the Hatch-Waxman's 14-year limit will adjust to the shorter FDA process. Nevertheless, the increased period of market exclusivity will be an incentive to develop new therapies.

Along with cutting approval time, discovery and development costs are predicted to decrease by $137 million dollars per drug. This will likewise provide further incentives for drug companies to attempt to bring new therapies to the marketplace.

VII. CONCLUSION

There are multiple opportunities and strategies to increase market exclusivity for a patent's term. There are also many possible pitfalls in evaluating the USPTO and FDA timelines. Timing is critical for the economic fate of small biotech companies developing novel therapies. A diligent and detailed patent prosecution team is necessary to balance the multiple USPTO and FDA concerns, while maximizing the opportunities to extend patent terms and market exclusivity.

* The passage of Greater Access to Affordable Pharmaceuticals Act (GAAPA) is still pending, which would strike out the third requirement for ANDA filing and eliminate the use of the ANDA blocking strategy mentioned above. Furthermore passage of this act would introduce a 30-day deadline to register patents with the FDA after approval, or be barred from civil actions for patent infringements. It is important that for a company to work with a patent prosecution team that is aware of the most current implications of statutory and judicial implications.

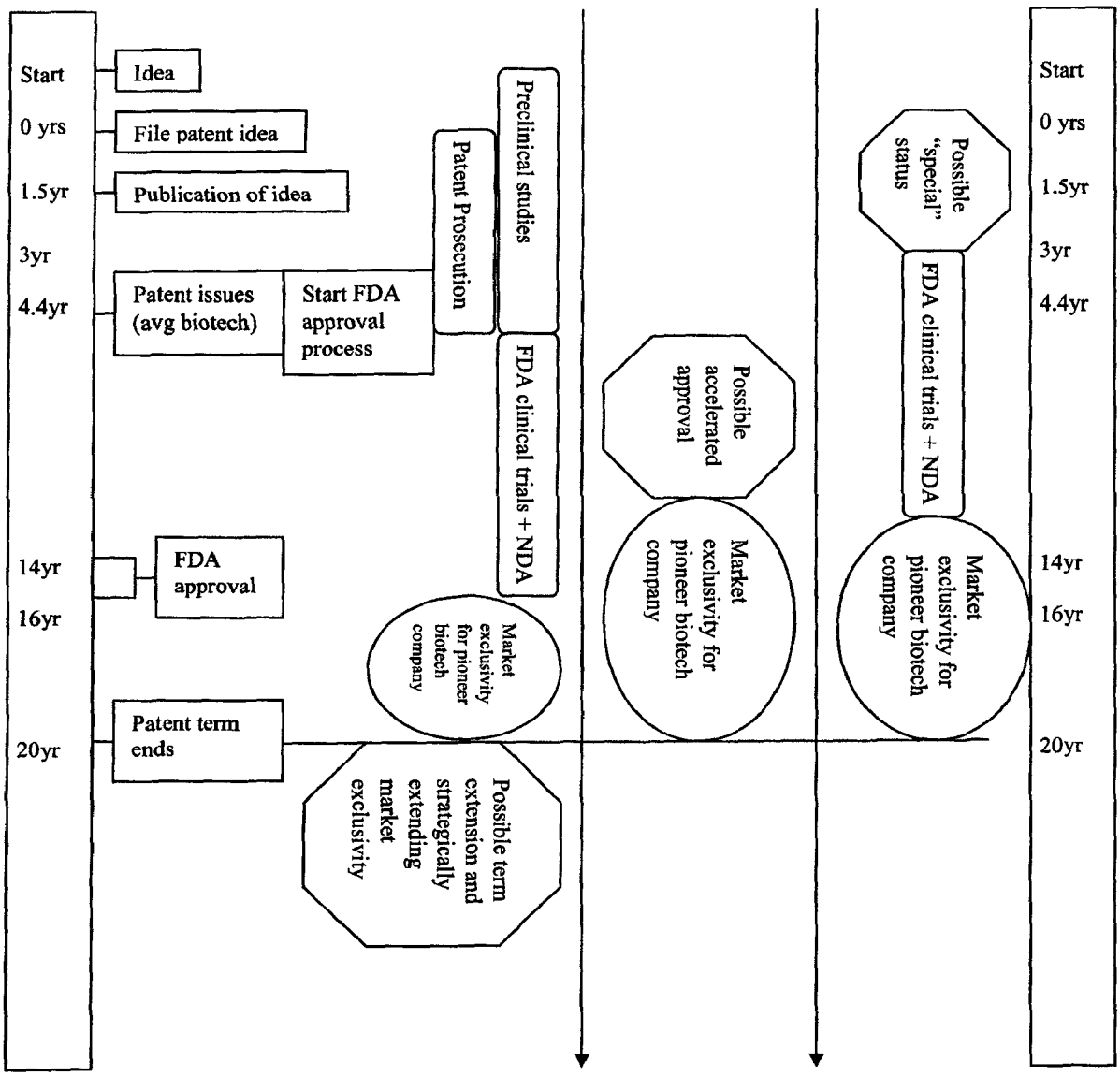
Start
0 yrs
1.5yr
3yr
4.4yr
14yr
16yr
20yr
Idea
File patent idea
Publication of idea
Patent issues (avg biotech)
Start FDA approval process
FDA approval
Patent term ends
Preclinical studies
Patent Prosecution
FDA clinical trials + NDA
Market exclusivity for pioneer biotech company
Possible term extension and strategically extending market exclusivity
Possible accelerated approval
Market exclusivity for pioneer biotech company
Possible "special" status
FDA clinical trials + NDA
Market exclusivity for pioneer biotech company
Start
0 yrs
1.5yr
3yr
4.4yr
14yr
16yr
20yr

Figure 1

LOSS OF PATENT RIGHTS –
"EXPERIMENTAL USE" VS. ON-SALE BAR/PUBLIC USE

Patents are often one of the most important assets a company possesses. The timing of patent filings is a crucial consideration for companies as new products are developed. Uniquely, the United States patent laws provide for a "first to invent" system (rather then a "first to file" system as in most foreign countries) and a one year grace period for a patent filing from the date of first public use or sale of the invention.

Despite the costs associated with bringing a new drug product to market and the consequences if the patents protecting that new drug product are later determined in litigation to be invalid, in many cases too little consideration is paid to events which take place during product development and the potential catastrophic results if a patent strategy has not been implemented to address those events.

One situation which consistently arises is the tension between presenting data in scientific conferences and/or publishing the same to promote scientific achievements or to enhance prospects of raising capital, and the potential loss of patent rights as a result of doing so. Activities seemingly as innocent as running clinical studies in support of a potential FDA filing can have far-reaching implications on the ability to obtain patent coverage encompassing the results of such testing, if sufficient care and attention is not paid to the development, timing and implementation of a patent strategy. The impact of clinical studies which occur more than one year prior to the filing date of patent applications may become a focal point concerning the validity of resulting patents particularly in view of a recent court decision. Activities seemingly as innocent as seeking a partner to commercialize a drug product may have similar implications, in certain situations. The issue to be considered is when those activities took place in comparison to when patent applications were filed.

What is the Law?

A patent claim is not valid if "the invention was... in public use or on sale in this country, more than one year prior to the date of the application for patent in the United States". *35 U.S.C. §102(b).* The fact that, e.g., the use was an experimental use, may be deemed by a court to negate public use in certain circumstances, but that experimental use must be deemed by the court to have perfected a feature in the patent claim(s) in question, or to have improved or verified a feature of the invention which is inherent that that claim(s). Recent court decisions confirm the fact that the one year grace period should not be relied on whenever possible, and certainly that activities that might be considered by the inventor and/or the assignee of the invention (hereinafter collectively referred to as "the inventive entity") to fall within exemptions to the §102(b) statutory bar need to be carefully scrutinized because such activities may later be deemed by a U.S. federal court to invalidate patent claims covering the invention.

The test which is now applied by the courts concerning whether an invention was in public use or on sale was set forth by the U.S. Supreme Court in *Pfaff v. Wells Electronics, Inc.*, 525 U.S. 55 (1998). The test articulated in *Pfaff* is whether, prior to the critical date (i.e., one year prior to the original filing date of the U.S. application), the claimed invention (i) was the subject of a commercial sale or offer for sale, or was publicly used by a person other then the inventor who is under no confidentiality obligation; and (ii) was ready for patenting.

The *Pfaff* court decision did not elaborate on what was meant by "a commercial offer for sale" (the first prong of its test), as it was clear in that case that a commercial offer had been made and accepted. In applying the ruling in *Pfaff*, the courts have generally construed that requirement to mean that the offer must meet the level of an offer for sale in the contract sense, to be analyzed under the law of contracts as generally understood. *Group One Ltd. v. Hallmark Cards, Inc.*, 254 F. 3d 1041 (Fed. Cir. 2001). It has further been construed to mean that activity which does not arise to the level of a

formal offer under contract law principles does not constitute a commercial offer for sale under *Pfaff*.

With respect to the second prong of the §102(b) bar test articulated in *Pfaff*, the Supreme Court in that case stated that the "ready for patenting" test may be satisfied in at least two ways: by proof of reduction to practice before the critical date; or by proof that prior to the critical date the inventor had prepared drawings or other descriptions of the invention that were sufficiently specific to enable a person skilled in the art to practice the invention.

The federal courts have now had an opportunity to apply the holding in *Pfaff* in a number of important pharmaceutical litigations, discussed below.

Clinical Trials May Constitute Invalidated Public Use

Recently, the Court of Appeals for the Federal Circuit ("CAFC") held that the clinical testing of a drug product does not qualify as an experimental use to negate the §102(b) bar where the tests were conducted to determine safety and efficacy, and did not involve the claimed features of the invention. *SmithKline Beecham v. Apotex,* 365 F. 3d 1306 (Fed. Cir. 2004)[1]. In this case, the United States District Court for the Northern District of Illinois considered whether claim 1 of U.S. Patent No. 4,721,723 (the '723 patent) owned by SmithKline would be infringed by Apotex' generic product. Claim 1 of the '723 patent recited in its entirety "crystalline paroxetine hydrocholoride hemihydrate." Following a bench trial, the court determined that the paroxetine hydrochloride anhydrate product produced by Apotex will not infringe claim 1. On appeal, the CAFC reversed the lower court's decision concerning non-infringement, but nevertheless determined that there was a public use bar under 35 U.S.C. §102(b) which rendered claim 1 of the '723 patent invalid.

[1] Decided April 23, 2004.

The pertinent facts considered on appeal are as follows. In May 1985, SmithKline began double-blind clinical trials in the United States to determine the safety and efficacy of paroxetine hydrochloride ("PHC") hemihydrate capsules to treat depression symptoms. These clinical trials occurred more than one year before SmithKline's October 23, 1985 filing date for the '723 patent. The CAFC determined that those clinical trials constituted a public use of the invention.[2] The CAFC then considered whether those tests qualified for the experimental use negation of the statutory public use bar. The CAFC determined that the claim on appeal (claim 1), which simply read "crystalline paroxetine hydrochloride hemihydrate," embraced the compound itself, without any further limitation regarding efficacy, commercial use, or pharmaceutical viability. *Id.* at 16. Consequently, the court found that the clinical tests in question (which measured the efficacy and safety of the compound as an antidepressant) did not involve testing concerning the claimed features of the invention, and concluded that the 1985 clinical tests did not qualify as an experimental use to negate the statutory bar, as these tests did not perfect a claimed feature of claim 1, nor did the testing improve or verify a feature of claim 1. *Id.* at 34. Mentioning the fact that only claim 1 was before it on appeal, the court also provided some insight into how the these same clinical trials might have met the experimental use negation of the §102(b) bar with respect to inventions claimed in the more specific claims of the '723 patent. Clearly, the court was hinting that claim 5 (which called for the pharmaceutical composition to have an "effective anti-depressant amount" of the hemihydrate) and claim 6 (which was a method of treatment of depression by administering the hemihydrate) might have met a different fate, because the language of those claims might be sufficiently connected to efficacy such that the clinical efficacy testing would have qualified for the experimental use negation of the §102(b) bar.

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[2] The *Pfaff* Court did not address the question of when a use is "public." The CAFC in *SmithKline* made the express assumption that the clinical trials were subject to satisfactory controls based on them by SmithKline, but nevertheless noted that the clinical trials were conducted without any apparent confidentiality restrictions on the patients or the administering physicians. This was an apparent nod to the CAFC's earlier decisions where it was stated that factors that are considered in determining whether a use is experimental include the nature of the clinical trials; and whether the participants were placed under any limitation or obligation of confidentiality. See, e.g., *Baker Oil Tools, Inc. v. Geo Vann, Inc.*, 828 F. 2d 1158, 1564 (Fed. Cir. 1987); *In re Brigance*, 792 F. 2d 1103, 1107-08 (Fed. Cir. 1986).

The On-Sale Bar

Consistently applied by the courts, it is not even necessary to any party to the use, sale or offer for sale recognize the patentable characteristics of the product at that time for the §102(b) bar to apply. For example, in *Abbott Laboratories v. Geneva Pharmaceuticals, et al.,* 182 F. 3d 1315 (Fed. Cir. 1999)[3], Abbott sued Geneva Pharmaceuticals, Novopharm Limited, and Invamed, Inc., for infringement of its U.S. Patent No. 5,504,207 after each of these companies filed an abbreviated new drug application (ANDA) seeking approval to market a generic version Abbott's hypertensive drug drug product, Hytrin® (terazosin hydrocholoride). Each of the generic products contained the Form IV anhydrate of Hytrin. Form IV was the subject matter of at least three commercial sales (the first prong of *Pfaff*) by a third party in the United States more then one year prior to the filing date of the '207 patent, and the CAFC held that the parties' ignorance to the fact that they were dealing with the Form IV anhydrate was irrelevant to meeting the commercial sale prong of *Pfaff*. The CAFC further held that it was clear that the invention was "ready for patenting" (the second prong of *Pfaff*) because the *third party* that had sold the Form IV had in turn bought the drug from two foreign manufacturers, who had already reduced it to practice. The court noted that there was no requirement that the sales offer specifically identify all the characteristics of an invention offered for sale or that the party recognizes the significance of all the characteristics at the time of the offer; if the product offered for sale inherently possesses each limitation of the claims, then the invention was "on sale." For these reasons, the CAFC affirmed the lower court's holding that the relevant claim of the '207 patent was invalid.

On the other hand, the CAFC has made it clear that only an offer which rises to the level of a commercial offer for sale in which the other party could make into a binding contract by simple acceptance constitutes an offer for sale under 102(b) which implicates the on-sale bar. In *Elan Corporation, PLC, v. Andrx Pharmaceuticals, Inc.*,

[3] Rehearing denied and rehearing on En Banc declined August 5, 1999, reported at: 1999 U.S. APP. LEXIS 19681; cert. denied January 19, 2000, reported at: 2000 U.S. LEXIS 169.

366 F. 3d 1336 (Fed. Cir. 2004)[4], Elan sued Andrx after Andrx submitted an ANDA seeking approval of a generic version of Elan's Naprelan® (once daily naproxen) formulation. More than the one year prior to Elan filing a patent application covering Naprelan, Elan had written letters to Lederle and other potential licensees offering to supply its once daily naproxen tablets. The issue before the court was whether Elan's letter to Lederle or any of its letters to potential licensees prior to the critical date contained "offers for sale". The CAFC held that the Lederle letter was not an offer to sell which implicated the §102(b) bar because the letter was "clear on its face that Elan was not offering to sale naproxen tablets to Lederle, but rather was offering a license under the patent and offering Lederle the opportunity to become its partner in the clinical testing in the eventual marketing of such tablet at some indefinite time in the future." *Id* at 14. Important to the court was the fact that the letter to Lederle lacked any mention of quantities, time of delivery, place of delivery, or product specifications beyond the general statement that a potential product would be a 500 mg once-daily tablet containing naproxen. Also, important was the fact that the Lederle letter did not include a sales price for sale tablets but rather referred to a "licensee fee." Because the CAFC held that there was no offer for sale, the court did not address the "ready for patenting" prong of the on-sale bar test.

Conclusion

The impact of early clinical trials on later filed patents may additionally be felt in view of the urging by various groups (including the American Medial Association and the International Committee of Medical Journal Editors) that it be required that all U.S. clinical trials are entered into a registry at their start so as to ensure that all clinical trial data is made public.[5] The gamut of activities associated with clinical studies (the studies themselves, presentation at scientific meetings, publication of articles and registry of the same), as well as other pre-commercialization activities (such as seeking marketing

[4] Decided May 5, 2004.

[5] Merck has now announced that it supports the idea of a government-run database. Other groups, such as the Pharmaceutical Research and Manufacturers Association of America, have expressed concerns (such as the risk of disclosing proprietary information to competitors, and whether the requirements would include not just drug makers, but also researchers who conduct drug tests).

partners, licensees, etc.) taking place prior to the filing of patent applications should be carefully considered in order to ensure compliance with 35 U.S.C. §102(b). Since the courts have focused on features of the patent claims with respect to §102(b) bar issues, patent claim strategies and patent filings should be developed early and matched with critical development dates in order to avoid a possible loss of patent rights.

By: Cliff Davidson, Esq.
Davidson, Davidson & Kappel, LLC
485 Seventh Avenue, New York, NY 10018
Telephone: (212) 736-1940
Facsimile: (212) 736-2427
Email: cdavidson@ddkpatent.com
www.ddkpatent.com

10.3 Antimetabolites

Antimetabolites *competitively* displace natural metabolism units (metabolites) or blocking enzymes and thus inhibit the metabolism and cell growth. Their action is substantially *non-specific*, i.e. the metabolism of *all* rapidly dividing cells is equally affected. For this reason antimetabolites are highly toxic, which substantially limits their use.

10.3.1. Folic acid antagonists

By a slight chemical modification of the folic acid, folic acid antagonists were obtained which have a substantially higher affinity to dihydrofolic acid reductase than folic acid itself and thus prevent the transfer of one-carbon fragments (see page 388). The consequence is a disturbed nucleic acid synthesis.

Aminopterin and *Methotrexate* were introduced into the therapy, of which aminopterin already had to be withdrawn from trade again.

Folic acid (citrovorum factor, Leucovorin®), but not folic acid, is an effective *antidote*.

Methotrexate (Methotrexate Bristol, Methotrexate "Lederle", Methotrexate Rhone-Poulenc) is preferably used in acute leukaemias, chorioepithelioma and various carcinomas, also in auto-immune diseases (see page 656).

The *dosing* to a high degree depends on the regimen. In the high doses sometimes used nowadays (1-20g) it is assumed that firstly the tumour cells are influenced by methotrexate and other body cells only later and it is thereby possible by timely administration of the antidote citrovorum factor to save the body cells from destruction (so-called citrovorum factor rescue).

The antineoplastic effect of such excessive methotrexate administrations is based on the fact that methotrexate in a high intercellular concentration is also then able to inhibit the low affine dihydrofolate reductase responsible for the resistance development.

10.3.2. Antagonists of purine and pyrimidine bases

Mercaptopurine and *tioguanin* belong to the purine analogues and *fluorouracil* and *cytarabine* belong to the pyrimidine analogues.

Mercaptopurine (6-mercaptopurine, Puri-Nethol®) can either be understood as an adenine or hypoxanthine analogue (replacement of the NH_2- of the adenine or the OH- group of the hypoxanthine by an SH- group). It works as a competitive inhibitor in purine biosynthesis. The intercellular active form is *6-mercaptopurine-ribonucleotide*. By inhibiting various enzymes, inter alia adenylosuccinate synthetase and phosphoribosylpyrophosphate amido transferase, the .....

684 Cytostatic Drugs

Table 21-1. Important Indications and Side Effects of Cytostatic Drugs

Cytostatic Drug	Main Indications	General Side Effects	Special Side Effects
a) lost derivatives	lymphosarcoma, lymphatic and myeloid leukaemia, Hodgkin's Disease, solid tumours of various organs, particularly ovarian, breast and bronchial carcinoma	early reactions: • nausea, vomiting • fever • shivering or sweating • abnormal fatigue • general lack of wellbeing	local intolerance damage to the efferent urinary tracts (all) mental disorders (ifosfamide) cardiac insufficiency (cyclophosphamide) gynecomastia (estramustine)
b) ethylanamines	similar to N-lost derivatives, retinoblastoma	disorder of the haematopoiesis: • anaemia • granulocytopenia • lymphopenia • thrombopenia	mental disorders, erythrodermia
c) alkyl sulfonate busulfan	leukaemia		liver damage
treosulfan	ovarian tumours		
d) nitrosourea derivatives	similar to N-lost derivatives, brain tumours (lomustine), melanoma, malignant melanoma (carmustine), prostate carcinoma	immune suppression disorder of the regeneration of the	functional disorders of the CNS, the kidneys and the liver, lung fibrosis

	(estramustine)	intestinal epithelium:	
e) cisplatin carboplatin	solid tumours of various organs	• aregeneratory enteropathy • stomatitis • enteritis • proctitis • malabsorption	Irreversible kidney damage, cardiac-circulatory and electrolyte metabolic disorders, peripheral neuropathies, hearing loss, sight disorders
f) dacarbazine	melanoma, sarcoma, lymphoma	hair growth disorder	liver vein closure, 'flu-like' symptoms, local intolerance, venous irritation
g) procarbazine	lymphoma	disorder of spermatogenesis and follicle maturation (ovulation)	mental disorders, MAO inhibition, alcohol intolerance, irreversible infertility
h) mitomycin	solid tumours of various organs		liver damage, kidney damage, lung damage
i) dactinomycin	rhabdomyosarcoma, Wilms' tumour, chorioepithelioma, amongst others	disorder of embryonic and foetal growth	local tissue damage
j) anthracyclines	leukaemia	hyperuricemia	cardiomyopathy; arrhythmia, heart failure, glycoside-

daunorubicin aclarubicin idarubicin			refractory myocardial failure (lethality 50%)
doxorubicin epirubicin	leukaemia, malignant lymphoma, solid tumours of various organs		
k) amsacrine	lymphatic and myelous leukaemia		functional disorders of the CNS, heart and liver, eye damage
l) mitoxantrone	leukaemia, malignant lymphoma, breast carcinoma		cardiomyopathy
m) methotrexate	lymphatic and myeloid leukaemia, chorioepithelioma, solid tumours of various organs, mycosis fungoides, psoriasis, non-Hodgkin's lymphoma		liver and kidney function disorders, lung function disorders, osteoporosis

UDK 615.277.3:577.184.161

Z.P. SOF'YINA, N.V. MYASISHCHEVA, F.G. ARSENYAN, A.M. YURKEVICH

POSSIBILITY OF POTENTIATING ANTINEOPLASTIC ACTION OF FOLIC ACID ANTAGONIST BY METHYLCOBALAMINE ANALOGUES

Cancer Research Center,
Academy of Medical Sciences (AMN), USSR, Moscow

Stimulating effect of cyanocobalamine on the growth of transplantable tumors in different types of animals (Rous chicken sarcoma, fibrosarcoma PW-2, sarcoma 45 and CCK in rats, Heren's carcinoma, sarcoma 180 and lymphosarcoma in mice) as well as weakening of the therapeutic effect of selected antitumor drugs when used in combination with vitamin B-12, as observed in the earlier studies, are caused by active biosynthesis of its coenzymes inside the animals' body. The assessment of the functional role of methylcobalamine (one of the cobalamine coenzymes involved in the processes of growth of normal and tumor cells) attracts the most attention.

Methylcobalamine is a coenzyme of methionine synthetase reaction, which is a key link determining the synergism of cobalamines and folic acid compounds activity in the cellular proliferation processes. The special significance of methylcobalamine for activation of this enzyme system was noted as a result of studying the deranged cobalamine exchanges in cases of human leucoses. Low efficiency of combined cytostatic therapy during certain forms of acute leucosis, proceeding with high blood concentrations of methylcobalamine, confirmed the specificity of its effect inside the body (N.V. Myasishcheva et al., 1969). At present, the active role of methylcobalamine in the processes of cellular proliferation of hematogenic tissue in healthy animals. Under the effect of methylcobalamine, the number of DNA-synthesizing cells in the spleen of mice as well as their mitotic activity and the proliferative pool value increase (O.D. Golenko et al.). The combined administration of methylcobalamine and endogenic blastomogens was found to cause significant increase in frequency of developing hemoblastoses in mice. The important element of the mechanism of stimulating action of cobalamines consists in their inducing effect on methionine synthetase activity. In the normal mammalian cell and human tumor cell cultures, methionine synthetase activity increases noticeably as the cobalamine content in the culturing medium goes up (Mangum et al.; Kamely et al.). Tumor cells of various types, however, differ from normal cells by their ability to potentiate methionine synthesis, required during intensive growth, under the effect of cobalamines (Halpern et al.; Chello and Bertino). The cobalamine-dependent methionine synthetase based rescue path likely represents the principal mechanism of developing leucotic cells tolerance towards methotrexate (MTX) by providing increase in the intracellular pool of tetrahydrofolic acid independent of the folate reductase system (N.V. Myasishcheva; Sauer and Jaenicke).

Therefore, it is realistic to expect potentiation of the antitumor effect of this metabolite by combining its application with cobalamine coenzyme antagonists. Understanding the mechanism of cobalamine effect laid the ground for targeted synthesis of methylcobalamine analogues and their testing as potential antitumor compounds.

In the chemotherapeutic experiments, difluoro-chloromethylcobalamine and methylcobalamine chloropalladate have been studied, which demonstrated activity during *in*

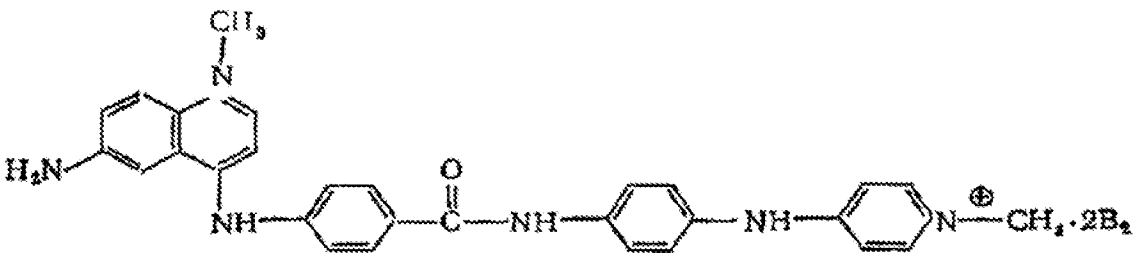
vitro studies in suppressing the growth of bacterial cells as well as inhibiting DNA synthesis in the human embryonic fibroblasts culture (N.V. Myasishcheva et al., 1977).

When developing the scheme of combined treatment, the following main aspects of physiological action of cobalamines inside the body were considered: control over delivery of the folic acid compounds to the cells and folate coenzyme formation, as well as intensity of cobalamine absorption by the tumor cells (Burke et al.; Tisman and Herbert; Floodh and Ullbegr). Based on this, one could have expected to see the selectivity of action of the studied compounds as well as a possibility of a decrease in activity of cobalamine-dependent enzyme inside the body. However, it was hard to expect a significant effect as a result of isolated application thereof. Therefore, it was important for us to evaluate the antitumor effect of these compounds against inhibition of dehydrofolate reductase activity using MTX.

M a t e r i a l s a n d m e t h o d s. The studies were conducted using mice of the following lines: $C_{57}BL$, CBA, BALB/c as well as hybrids $BDF_1/C_{57}BL_6$ DBA(2), weighing 20-25 g, obtained from the nursery of the USSR Academy of Medical Sciences (AMN). Antitumor activity of methylcobalamine analogues was studied using transplantable leucoses L-1210 and La, as well as solid tumors: adenocarcinoma of the mammary gland (Ca-755), carcinoma of the uterine cervix (CUC-5) and carcinoma of the intestine (ACATOL). We have chosen solid tumors as the main object of investigation, since it is easier to establish a stimulating effect of methylcobalamine using these tumors as compared to the leucosis models L-1210 and La in mice, with high proliferative pool and quite short life span of the animals.

Methylcobalamine (CH_3Cbl) and difluoro-chloromethylcobalamine (CF_2ClCbl) were obtained using a known method (Wood et al., 1968) modified in the extraction section (Ye.M. Tachkova et al.). Methylcobalamine chloropalladate ($MetCbl \bullet PdCl_3$) was synthesized using Ye.G. Chauser's method. Methylcobalamine was administered intramuscularly in the dose of 10 μg/kg twice over the course of treatment with the interval of 96 hours, CF_2ClCbl was administered on a daily basis subcutaneously in the dose of 500 mg/kg instantly or twice a day in the dose of 250 mg/kg for five days. Poorly soluble methylcobalamine chloropalladate was administered perorally in 2% starch suspension in the daily dose of 500 mg/kg for 5 days or twice with the interval of 96 hours. The daily dose was administered instantly or at 250 mg/kg twice a day. MTX manufactured by "Lederle" was used in the dose of 10 mg/kg intraperitoneally with the interval of 96 hours.

In our studies, the activity of cobalamine derivatives was studied not only in combination with MTX, but also with quinoline derivative (NSC-176319):



The compound was obtained from the U.S. National Cancer Institute based on the collaboration agreement between USSR and USA in the field of tumor chemotherapy. According to the characteristics provided by the American scientists, the compound represents a methionine synthetase inhibitor (Carter et al.). Quinoline derivative was applied intraperitoneally in the dose of 5 mg/kg daily or with the interval of 96 hours, which is a half of the maximum tolerable dose for utilized regime. The treatment began 48 hours after tumor transplantation. The results of treatment were evaluated 24 hours upon completion of the course of treatment as well as at different points throughout the life span of the animals. A percentage

of inhibition of the tumor growth, calculated based on the conditional volume, as well as increase in life span of the animals were used as efficiency criteria. In each of the tests, the control and experimental groups were designed in such a way that their population provided statistical significance of the minimal accountable percentages of inhibition of tumor growth (50%) and increase in life span of mice (25%). According to the specified requirements, the experimental groups consisted of 6 – 10 mice, while control groups included 6 – 13 animals, depending on utilized tumor strain.

Results and discussion. In the course of conducted studies it was found for the first time that methylcobalamine has a stimulating effect on the growth of transplantable tumors Ca-755, ACATOL, and to the lesser extent – CUC-5 (Table 1). The maximum intensity of tumor growth under the effect of methylcobalamine was observed during transplantation of Ca-755 to hybrid mice BDF_1 (180%) as compared to the growth of the same tumor in mice of the pure B_{57}-Bl line. The stimulation of tumor cell duplication occurred during the period of methylcobalamine administration; the maximum difference in tumor size between the animals of the experimental and control groups was observed directly upon completion of drug administration. Later on, the tumor growth in mice administered with methylcobalamine has slowed down. In case of ACATOL transplantation to mice of different gender, the intensity of tumor growth under the effect of methylcobalamine varied. The stimulating action of the drug was more pronounced in the male animals (see Table 1).

As expected, an isolated effect of methylcobalamine analogues caused insignificant inhibition of the growth of transplantable tumors Ca-755 and CUC-5, and only directly upon administration of the drugs (Table 2).

During comparative evaluation, the maximum inhibiting activity was established when utilizing methylcobalamine chloropalladate. The inhibition efficiency of Ca-755 growth was more pronounced in hybrid mice BDF_1 as compared to $B_{57}Bl$ mice. As mentioned earlier, the stimulating action of methylcobalamine was much more pronounced specifically in BDF_1 mice. In this series of experiments, the life span of BDF_1 mice with adenocarcinoma of the mammary gland, treated with CF_2ClCbl and methylcobalamine chloropalladate, increased by 50% (see Table 2). At the same time, no ACATOL growth inhibition effect was observed when administering methylcobalamine derivatives. A considerable difference was found in the effect of methylcobalamine derivatives on the tumor depending on their application regime (see Table 2). It is likely that in case of one-time administration of a large dose (500 mg/kg), a dissociation of the drug may occur followed by subsequent formation of the active form stimulating the tumor growth.

According to our assumption, when combining methylcobalamine analogues with MTX, their effect on the tumor increases (Ca-755, CUC-5; Table 3). An increase in antitumor effect as a result of combined treatment was observed directly after the drug administration, and specifically during the subsequent period: the percentage of tumor growth inhibition remained quite high when there was no more effect from MTX along.

In order to understand the possible operating mechanism of methylcobalamine analogues in the animals' body, a comparative analysis was conducted to compare the growth of similar strains under condition of isolated effect of methionine synthetase inhibitor (quinoline derivative) and its combined effect with MTX. The inhibition of growth of Ca-755, CUC-5 and ACATOL increased depending on drug concentration. The drug affected Ca-755 most effectively. When the dose was increased from 5 to 15 mg/kg, the inhibition of tumor growth increased to 40 and 96% respectively.

Table 1.

Methylcobalamine effect on the growth of selected transplantable tumors

Tumor	Drug dose, μg/kg	Drug administration schedule upon transplantation of tumor, days	Tumor growth upon drug administration, % vs. control		
			1 day	7 days	14 days
Ca-755 $C_{57}Bl$	10	2^{nd} and 6^{th}	+74	+21	+23
BDF_1	10	2^{nd} and 6^{th}	+180	+65	+10
ACATOL:	10	2^{nd} and 6^{th}	+20	+23	+31
females					
males	10	2^{nd} and 6^{th}	+126	+37	+33

N o t e. Here and in Tables 2 – 6, the "plus" sign denotes stimulation of tumor growth.

Table 2.

Antitumor effect of methylcobalamine analogues

Tumor	Drug	Drug dose, μg/kg	Drug administration schedule upon transplantation of tumor, days	Inhibition of tumor growth, % vs. control			Increase in life span of mice, % vs. control
				1 day	7 days	15 days	
Ca-755	Defluoro-chloromethyl-cobalamine (CF_2ClCbl)	250 + 250	2^{nd} – 6^{th}	30	+8		54
		250 + 250	2^{nd} – 6^{th}	43	38	0	16
CUC-5		250 + 250	2^{nd} – 6^{th}	0	0	0	0
ACATOL							
Ca-755	Trichloromethyl-cobalamine complex with ($MetCb•PdCl_3$)	250 + 250	2^{nd} – 6^{th}	90	59		50
(BDF_1)		500	2^{nd} – 6^{th}	13	16	20	
		250 + 250	2^{nd} – 6^{th}	80	23	0	10
CUC-5		500	2^{nd} – 6^{th}	+130	+15	+18	0
ACATOL		250 + 250	2^{nd} – 6^{th}	0	0	0	0

However, along with increase in drug dosage, there was a noticeable increase in its toxicity. For example, in case of leucosis strains L-1210 and La, the most optimal dose, based on our data, was 10 mg/kg, which resulted in 3-4 times increase in life span of the animals. In case of reduced dose, the drug treatment effect on mice with leucoses was significantly lower. In case of solid tumors, no significant increase in life span of mice was observed in our studies. In case of combined administration of the drug with MTX even in low doses (5 mg/kg), a summation of the effects was observed, which confirmed the increase in tumor growth inhibition rate (Table 4). In case of delayed beginning of treatment of animals (day 8 after tumor transplantation) and daily administration of the drugs for 5 days (5 mg/kg of quinoline derivative; 2 mg/kg MTX), the results were even more demonstrative (Ca-755), however, as a result of combined treatment, the overall toxicity increased as well (Table 5).

Increase in tumor growth inhibition rate as well as in the life span of the animals was observed during combined treatment using methylcobalamine chloropalladate and quinoline derivative (NSC-176319; Table 6). Considering potentiation of MTX effect when used in combination with methylcobalamine analogues and methionine synthetase inhibitor, we have conducted combined treatment of mice with Ca-755 using all 3 inhibitors: MTX, quinoline

Table 3.

Antitumor effect of the combination of MTX and methylcobalamine analogues

Tumor	Drug	Drug dose, mg/kg	Drug administration schedule upon transplantation of tumor, days	Inhibition of tumor growth, % vs. control					Increase in life span of mice, % vs. control
				1 day	5 days	7 days	10 days	14 days	
Ca-755 ($C_{57}BL$)	MTX	10	2nd and 6th	75		10	+32		16
	$MetCbl•PdCl_3$	250 + 250	2nd and 6th	58		20	14		0
	MTX + $MetCbl•PdCl_3$	10 +250 + 250 (administered at the same time)	2nd – 6th	97		75	0		0
CUC-5 (CBA)	MTX	10	2nd and 6th	90		48		40	0
	$MetCbl•PdCl_3$	500	2nd and 6th	+220		+100		+80	0
	MTX + $MetCbl•PdCl_3$	10 + 500 (administered at the same time)	2nd and 6th	97		65		40	40
Ca-755 (hybrids)	MTX	10	2nd and 6th	87	81	45	67		
	CF_2ClCbl	500	2nd and 6th	+67	+5	+21	5		
	MTX + CF_2ClCbl	10 + 500 (CF_2ClCbl was administered 3 hr prior to MTX)	2nd and 6th	97	99	74	67		

Table 4.

Effect of the combined application of MTX and NSC-176319 on tumors in mice

Tumor	Drug	Drug dose, mg/kg	Drug administration schedule upon transplantation of tumor, days	Inhibition of tumor growth, % vs. control				
				1 day	5 days	7-8 days	10 days	14-16 days
Ca-755 (BDF_1)	MTX	5	2^{nd} and 6^{th}	46	9	19	19	23
	NSC-176319	5	2^{nd} and 6^{th}	18	8	41	29	30
	MTX + NSC-176319	5 + 5 (administered at the same time)	2^{nd} and 6^{th}	81	62	66	43	31
CUC-5 (CBA)	MTX	10	2^{nd} and 6^{th}	69		74		85
	NSC-176319	10	2^{nd} and 6^{th}	20		55		31
	MTX + NSC-176319	10 + 10 (administered at the same time)	2^{nd} and 6^{th}	88		84		75
ACATOL (BALB/c)	MTX	10	2^{nd} and 6^{th}	45	53	44		
	NSC-176319	5	2^{nd} and 6^{th}	12	27	30		
	MTX + NSC-176319	10 + 5 (MTX was administered 20 min after NSC-176319)	2^{nd} and 6^{th}	65	43	40		

Table 5.

Effect of MTX and NSC-176319, applied as a complex, on the growth of Ca-755 in mice

Drug	Drug dose, mg/kg	Drug administration schedule upon transplantation of tumor, days	Inhibition of tumor growth, % vs. control		Ratio between dead animals and the number of animals in the group
			1 day	3 days	
MTX	2	8 – 12	12	+13	1/6
NSC-176319	5	8 – 12	12	+8	0/6
MTX + NSC-176319	2 + 5 (administered at the same time)	8 – 12	76	79	5/6

Table 6.

Effect of MTX, NSC-176319 and a complex of trichloromethylcobalamine with Palladium on the growth of Ca-755 in mice

Drug	Drug dose, mg/kg	Drug administration schedule upon transplantation of tumor, days	Inhibition of tumor growth, % vs. control			Increase in life span, % vs. control
			2 days	8 days	14 days	
MTX	10	2nd and 6th	99	51	0	14
NSC-176319	5	2nd and 6th	37	7	+29	0
MetCbl•$PdCl_3$	250	2nd and 6th	75	40	13	0
NSC-176319 + MetCbl•$PdCl_3$	5 + 250 (administered at the same time)	2nd and 6th	90	58	4	23
MetCbl•$PdCl_3$ + MTX	250 + 10 (MTX was administered 20 min after MetCbl•$PdCl_3$)	2nd and 6th	99	58	5	8
NSC-176319 + MTX	5 + 10 (MTX was administered 20 min after NSC-176319)	2nd and 6th	99	88	44	0
NSC-176319 + MetCbl•$PdCl_3$ + MTX	5 + 250 + 10 (NSC-176319 and MetCbl•$PdCl_3$ were administered at the same time, and MTX – in 20 min thereafter)	2nd and 6th	00 1	95	85	20

derivative and methylcobalamine chloropalladate, which is the most active analogue of cobalamine coenzyme (see Table 6).

As a result of combined application of methionine synthetase and dehydrofolate reductase inhibitors, the antitumor effect has increased significantly, especially in the long term upon completion of treatment. Under such conditions, 2 weeks after the drug administration was completed, the tumor growth inhibition rate was 85%, while in the groups of mice receiving each of the studied drugs individually or in combination of 2 drugs, no practical inhibition of tumor growth during these time periods was seen. However, it should be noted that at the same time the toxicity has increased as well. It was also established that the effect of drug combination changes significantly depending on the sequence of combinant administration and time intervals between them. Thus, simultaneous administration of NSC-176319 and MTX appeared to be significantly less toxic for the body, than administration thereof with the time interval of 3 hours, while the antitumor effect was the same.

Hence, the results of experimental studies support our assumption about a possibility of potentiation of antitumor effect of MTX using methylcobalamine analogues and methionine synthetase inhibitor. This opens up a new approach to therapeutic treatment of tumors using antagonists of the physiological regulator of folic acid compounds exchange inside the body. We have established an antitumor activity of cobalamine coenzyme antagonist. However, the activity of the studied methylcobalamine analogues blocking certain metabolic links is insufficient for complete and prolonged inhibition of tumor growth. The antitumor effect of cobalamine coenzyme analogues can be significantly potentiated by their combined application with MTX. Obtained experimental data suggests that it could be feasible to perform clinical trials to determine efficiency of such type of combinations. At present, our main task is to develop an optimal regime of combined treatment of tumors using specified drugs based on thorough analysis of the mechanism of their combined action inside the body.

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POSSIBILITY OF POTENTIATING ANTINEOPLASTIC ACTION OF FOLIC ACID ANTAGONIST BY METHYLCOBALAMINE ANALOGUES

Z.P. Sof'yina, N.V. Myasishcheva, F.G. Arsenyan, A.M. Yurkevich

S u m m a r y. The effect of methylcobalamine and its analogues (difluorochloromethylcobalamine – CF_2ClCbl and methylcobalamine chloropalladate - $MetCbl \bullet PdCl_3$) on the growth of transplantable tumors in mice: adenocarcinoma of the mammary gland (Ca-755), carcinoma of the uterine cervix (CUC-5), carcinoma of the intestine (ACATOL) was studied. The activity of the cobalamine coenzyme analogues was investigated when used alone or combined with inhibitors of dehydrofolate reductase and methionine synthetase. The results of the experiments indicate a stimulating effect of methylcobalamine on the growth of transplantable solid tumors in the animal organism. The antitumor activity of the methylcobalamine analogues studied was found to be higher in combined application with methotrexate. The most effective inhibition of tumor growth and the longer survival of the animals were achieved in combined application of methylcobalamine with methotrexate and methionine synthetase inhibitor, depending upon the scheme of administration.

CONTENTS

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З. П. СОФЬИНА, Н. В. МЯСИЩЕВА, Ф. Г. АРСЕНЯН, А. М. ЮРКЕВИЧ

ВОЗМОЖНОСТЬ УСИЛЕНИЯ ПРОТИВООПУХОЛЕВОГО ДЕЙСТВИЯ АНТАГОНИСТА ФОЛИЕВОЙ КИСЛОТЫ АНАЛОГАМИ МЕТИЛКОБАЛАМИНА

Онкологический научный центр АМН СССР, Москва

Стимулирующее действие цианокобаламина на рост перевиваемых опухолей разного вида животных (саркома кур Рауса, фибросаркома PW-2, саркома 45 и ССК крыс, карцинома Герена, саркома 180 и лимфосаркома мышей) и ослабление лечебного действия некоторых противоопухолевых препаратов при совместном их применении с витамином B_{12}, отмечаемые в ранних исследованиях, обусловлены активным биосинтезом его коферментов в организме животных. Оценка функциональной роли метилкобаламина — одного из кобаламиновых коферментов в процессах роста нормальных и опухолевых клеток — привлекает наибольшее внимание.

Метилкобаламин является коферментом метионинсинтетазной реакции — ключевого звена, определяющего синергизм действия кобаламинов и соединений фолиевой кислоты в процессах клеточной пролиферации. Особая значимость метилкобаламина для активации этой ферментной системы отмечена в результате изучения нарушенного обмена кобаламинов при лейкозах человека. Малая эффективность комбинированной цитостатической терапии при определенных вариантах острого лейкоза, протекающих с высокой концентрацией метилкобаламина в крови, подтверждала специфичность его действия в организме (Н. В. Мясищева и соавт., 1969). В настоящее время установлена активная роль метилкобаламина в процессах пролиферации клеток кроветворной ткани здоровых животных. Под воздействием метилкобаламина в селезенке мышей возрастают число клеток, синтезирующих ДНК, их митотическая активность и величина пролиферативного пула (О. Д. Голенко и соавт.). Обнаружено значительное увеличение частоты развития гемобластозов у мышей при комбинированном введении метилкобаламина с эндогенными бластомогенами. Важным моментом механизма стимулирующего действия кобаламинов является их индуцирующее влияние на активность метионинсинтетазы. В культурах нормальных клеток млекопитающих и опухолевых клеток человека активность метионинсинтетазы заметно возрастает с увеличением содержания кобаламинов в среде культивирования (Mangum и соавт.; Kamely и соавт.). Опухолевые клетки разного типа, однако, отличны от нормальных по своей способности под воздействием кобаламинов усиливать биосинтез метионина, необходимый при интенсивном росте (Halpern и соавт.; Chello и Bertino). Спасательный путь с помощью кобаламинзависимой метионинсинтетазы, обеспечивая увеличение внутриклеточного пула тетрагидрофолиевой кислоты независимо от фолатредуктазной системы, представляет, по-видимому, основной механизм развития устойчивости лейкозных клеток к метотрексату (МТХ) (Н. В. Мясищева; Sauer и Jaenicke).

В связи с этим реальна возможность усиления противоопухолевого эффекта данного метаболита путем его комбинированного применения

с антагонистами кобаламинового кофермента. Понимание механизма действия кобаламинов послужило обоснованием для направленного синтеза аналогов метилкобаламина и их испытания в качестве потенциальных противоопухолевых соединений.

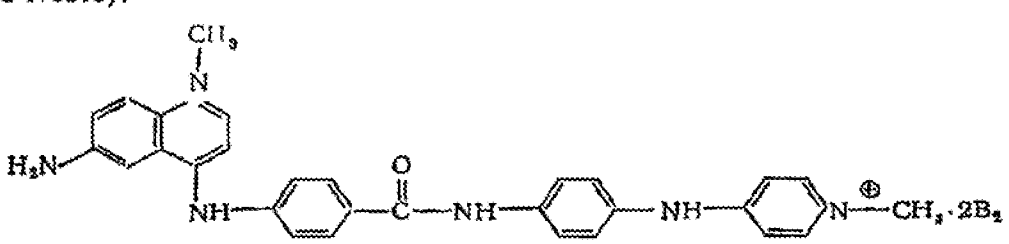
В химиотерапевтических экспериментах были изучены дифторхлорметилкобаламин и хлорпаллодат метилкобаламина, обнаруживавшие активность при исследованиях in vitro в подавлении роста бактериальных клеток и торможении синтеза ДНК в культуре эмбриональных фибробластов человека (Н. В. Мясищева и соавт., 1977).

При разработке схемы комбинированного воздействия были учтены основные аспекты физиологического действия кобаламинов в организме: контроль за поступлением соединений фолиевой кислоты в клетки и образованием коферментов фолата, а также интенсивность поглощения кобаламинов опухолевыми клетками (Burke и соавт.; Tisman и Herbert; Floodh и Ullberg). В связи с этим можно было рассчитывать на избирательность действия исследуемых соединений и возможность снижения активности кобаламинозависимого фермента в организме. Однако трудно было ожидать значительного эффекта при их изолированном применении. Поэтому нам представлялось важным оценить противоопухолевое действие этих соединений на фоне торможения активности дигидрофолатредуктазы с помощью МТХ.

Материал и методы. Исследования проведены на мышах линии $C_{57}BL$, CBA, BALB/c и гибридах $BDF_1/C_{57}BL \times DBA(2)$, массой 20—25 г, полученных из питомника АМН СССР. Противоопухолевая активность аналогов метилкобаламина изучена на перевиваемых лейкозах L-1210 и La и солидных опухолях: аденокарциноме молочной железы (Са-755), раке шейки матки (РШМ-5) и аденокарциноме кишечника (АКАТОЛ). Мы выбрали в качестве основного объекта исследования солидные опухоли, на которых легче выявить стимулирующее влияние метилкобаламина, чем на модели лейкозов мышей L-1210 и La, с высоким пролиферативным пулом и весьма короткой продолжительностью жизни животных.

Метилкобаламин (CH_3Cbl) и дифторхлорметилкобаламин (CF_2ClCbl) получены по известному методу (Wood и соавт., 1968), модифицированному в разделе выделения (Е. М. Тачкова и соавт.). Хлорпаллодат метилкобаламина ($MetCbl \cdot PdCl_2$) синтезирован способом Е. Г. Чаусера. Метилкобаламин вводили внутримышечно из расчета 10 мкг/кг 2 раза на курс лечения с интервалом 96 ч, CF_2ClCbl — ежедневно подкожно из расчета 500 мг/кг одномоментно либо 2 раза в день по 250 мг/кг в течение 5 дней. Плохо растворимый хлорпаллодат метилкобаламина вводили перорально в 2% крахмальной суспензии в суточной дозе 500 мг/кг в течение 5 дней или 2 раза с интервалом 96 ч. Суточная доза вводилась одномоментно или по 250 мг 2 раза в день. МТХ фирмы «Lederle» использовали в дозе 10 мг/кг внутрибрюшинно с интервалом 96 ч.

В наших исследованиях активность кобаламиновых производных изучена не только при комбинированном применении с МТХ, но также с хинолиновым производным (NSC-176319):



Препарат получен нами из Национального института рака США в соответствии с соглашением о сотрудничестве между СССР и США в области химиотерапии опухолей. Согласно характеристике, представленной американскими учеными, препарат является ингибитором метионинсинтетазы (Carter и соавт.). Хинолиновое производное применяли внутрибрюшинно в дозе 5 мг/кг ежедневно или с интервалом 96 ч, что составляет половину максимально переносимой дозы для использованного режима. Лечение начинали через 48 ч после перевивки опухоли. Результаты воздействия оценивали через 24 ч после окончания курса лечения и в различные сроки на протяжении жизни животных. Критерием эффективности служили процент торможения роста опухоли, вычисляемой по условному объему, и увеличение продолжительности жизни животных. В каждом из опытов контрольные и опытные группы составляли так, чтобы их численность обеспечивала статистическую значимость минимальных учитываемых процентов торможения

роста опухолей (60%) и увеличения продолжительности жизни мышей (25%). В соответствии с указанными требованиями опытные группы состояли из 6—10 мышей, а контрольные — из 6—13 животных, в зависимости от используемого штамма опухоли.

Результаты и их обсуждение. В проведенных исследованиях впервые обнаружено стимулирующее влияние метилкобаламина на рост перевиваемых опухолей Са-755, АКАТОЛ, в меньшей степени — на рост РШМ-5 (табл. 1). Наибольшая интенсивность роста опухоли под воздействием метилкобаламина наблюдалась при перевивке Са-755 мышам-гибридам BDF_1 (180%) по сравнению с ростом той же опухоли у мышей чистой линии $C_{57}Bl$. Стимуляция размножения опухолевых клеток происходила в период введения метилкобаламина; наибольшее различие в величине опухолей у животных опытной и контрольной групп выявлено непосредственно после окончания введения препарата. В последующие сроки рост опухолей у мышей, получавших метилкобаламин, замедлялся. При перевивке АКАТОЛ мышам разного пола интенсивность роста опухоли при воздействии метилкобаламина различна. Стимулирующее действие препарата было значительнее выражено у самцов (см. табл. 1).

Как и следовало ожидать, изолированное воздействие аналогов метилкобаламинов тормозило рост перевиваемых опухолей Са-755, РШМ-5 в небольшой степени и лишь непосредственно после введения препаратов (табл. 2).

При сравнительной оценке наибольшая ингибирующая активность обнаружена при использовании хлорпаллодоата метилкобаламина. Эффективность торможения роста Са-755 была более выражена у мышей-гибридов BDF_1 по сравнению с мышами $C_{57}Bl$. Как было указано, именно у мышей BDF_1 в значительно большей степени проявлялось и стимулирующее действие метилкобаламина. В этой серии опытов продолжительность жизни мышей BDF_1 с аденокарциномой молочной железы при воздействии CF_2ClCbl и хлорпаллодата метилкобаламина увеличивалась на 50% (см. табл. 2). В то же время при введении производных метилкобаламина отсутствовал эффект торможения роста АКАТОЛ. Отмечено большое различие в действии кобаламиновых производных на опухоль в зависимости от режима их применения (см. табл. 2). По-видимому, при однократном введении большой дозы (500 мг/кг) возможна диссоциация препаратов с последующим образованием активной формы, стимулирующей рост опухоли.

В соответствии с нашим предположением при комбинировании аналогов метилкобаламина с МТХ обнаружено усиление их действия на опухоль (Са-755, РШМ-5; табл. 3). Увеличение противоопухолевого эффекта в результате комбинированного воздействия проявлялось непосредственно после курса введения препаратов и, особенно, в последующий период: когда эффект действия одного МТХ уже отсутствовал, сохранялся достаточно высокий процент торможения роста опухоли.

Таблица 1

Влияние метилкобаламина на рост некоторых перевиваемых опухолей

Опухоль	Доза препарата, мкг/кг	Срок введения препарата после прививки опухоли, дни	Рост опухоли после введения препарата, % к контролю		
			1 день	7 дней	14 дней
Са-755 $C_{57}BL$	10	2-й и 6-й	+74	+21	+23
BDF_1	10	2-й и 6-й	+180	+65	+10
АКАТОЛ:	10	2-й и 6-й	+20	+23	+31
самки					
самцы	10	2-й и 6-й	+126	+37	+33

Примечание. Здесь и в табл. 2—6 знак «плюс» обозначает стимуляцию роста опухоли.

Таблица 2

Противоопухолевое действие аналогов метилкобаламина

Опухоль	Препарат	Доза препарата, мкг/кг	Срок введения препарата после прививки опухоли, дни	Торможение роста опухоли, % к контролю			Увеличение продолжительности жизни мышей, % к контролю
				1 день	7 дней	15 дней	
Са-755	Хлордифторметилкобаламин (CF_2ClCbl)	250+250	2—6-й	30	+8		54
		250+250	2—6-й	43	38	0	16
РШМ-5		250+250	2—6-й	0	0	0	0
АКАТОЛ							
Са-755	Комплекс трихлорметилкобаламина с ($MetCb \cdot PdCl_2$)	250+250	2—6-й	90	59		50
(BDF_1)		500	2—6-й	13	16	20	
		250+250	2—6-й	80	23	0	10
РШМ-5		500	2—6-й	+130	+15	+18	0
АКАТОЛ		250+250	2—6-й	0	0	0	0

Для понимания возможного механизма действия аналогов метилкобаламина в организме животных был осуществлен сравнительный анализ роста тех же опухолевых штаммов при изолированном влиянии ингибитора метионинсинтетазы — хинолинового производного — и его сочетанного воздействия с МТХ. Торможение роста Са-755, РШМ-5 и АКАТОЛ увеличивалось в зависимости от концентрации препарата. Наиболее эффективно препарат воздействовал на Са-755. При увеличении дозы от 5 до 15 мг/кг торможение роста опухоли возрастало соответственно до 40 и 96%. Однако с увеличением дозы препарата заметно возрастала и его токсичность. Например, при штаммах лейкозов L-1210 и La наиболее оптимальной дозой, по нашим данным, являлась доза 10 мг/кг, при которой в 3—4 раза увеличивалась продолжительность жизни животных. При уменьшении дозы эффект воздействия препарата на мышей с лейкозами был существенно ниже. При солидных опухолях в наших исследованиях не было отмечено значительного увеличения продолжительности жизни мышей. При сочетанном введении препарата с МТХ даже в малой дозе (5 мг/кг) наблюдалась суммация эффекта, что подтверждало увеличение торможения роста опухоли (табл. 4). При более позднем начале лечения животных (на 8-й день после перевивки опухоли) и ежедневном введении препаратов в течение 5 сут (5 мг/кг хинолинового производного; 2 мг/кг МТХ) результаты были еще более демонстративны (Са-755), но при суммарном воздействии увеличивалась также и общая токсичность (табл. 5).

Увеличение торможения роста опухоли и продолжительности жизни животных отмечено при комбинированном воздействии хлорпаллодата метилкобаламина и хинолинового производного (NSC-176319; табл. 6). Учитывая усиление действия МТХ при его комбинированном использовании с аналогами метилкобаламина и ингибитором метионинсинтетазы, мы осуществили комбинированное лечение мышей с Са-755 с применением всех 3 ингибиторов: МТХ, хинолинового производного и наиболее активного аналога кобаламинового кофермента — хлорпалладата метилкобаламина (см. табл. 6).

В результате комбинированного применения ингибиторов метионинсинтетазы и дигидрофолатредуктазы значительно усиливалось противоопухолевое действие, особенно в отдаленные сроки после окончания лечения. В этих условиях через 2 нед после окончания введения препаратов торможение роста опухоли составляло 85%, в то время как в группах мышей, получавших каждое из исследуемых соединений изолированно или комбинацию из 2 препаратов, в эти сроки угнетение роста

Таблица 3

Противоопухолевое действие комбинации МТХ и аналогов метилкобаламина

Опухоль	Препарат	Доза введения препаратов, мг/кг	Срок введения препаратов после прививки, дни	Торможение роста опухоли, % к контролю					Увеличение продолжительности жизни мышей, % к контролю
				1 день	5 дней	7 дней	10 дней	14 дней	
Са-755 (C_1,BL)	МТХ	10	2-й и 6-й	75		10	+32		16
	MetCbl·$PdCl_2$	250+250	2-й и 6-й	58		20	14		0
	МТХ+MetCbl·$PdCl_2$	10+250+250 (вводились одновременно)	2—6-й	97		75	0		0
РШМ-5 (CBA)	МТХ	10	2-й и 6-й	90		48		40	0
	MetCbl·$PdCl_2$	500	2-й и 6-й	+220		+100		+80	0
	МТХ+MetCbl·$PdCl_2$	10+500 (вводились одновременно)	2-й и 6-й	97		65		40	40
Са-755 (гибриды)	МТХ	10	2-й и 6-й	87	81	45	67		
	CF_2ClCbl	500	2-й и 6-й	+67	+5	+21	5		
	МТХ+CF_2ClCbl	10+500 (CF_2ClCbl, вводился за 3 ч до МТХ)	2-й и 6-й	97	99	74	67		

Таблица 4

Действие комбинированного применения МТХ и NSC-176319 на опухоли мышей

Опухоль	Препарат	Доза препаратов, мг/кг	Срок введения препарата после прививки опухоли, дни	Торможение роста опухолей, % к контролю				
				1 день	5 дней	7—8 дней	10 дней	14—16 дней
Са-755 (BDF_1)	МТХ	5	2-й и 6-й	46	9	19	19	23
	NSC-176319	5	2-й и 6-й	18	8	41	29	30
	МТХ+NSC-176319	5+5 (вводились одновременно)	2-й и 6-й	81	62	66	43	31
РШМ-5 (CBA)	МТХ	10	2-й и 6-й	69		74		65
	NSC-176319	10	2-й и 6-й	20		55		31
	МТХ+NSC-176319	10+10 (вводились одновременно)	2-й и 6-й	88		84		75
АКАТОЛ (BALB/c)	МТХ	10	2-й и 6-й	45	53	44		
	NSC-176319	5	2-й и 6-й	12	27	30		
	МТХ+NSC-176319	10+5 (МТХ вводился через 20 мин после NSC-176319)	2-й и 6-й	65	43	40		

Таблица 5

Действие МТХ и NSC-176319, применяющихся в комплексе, на рост Са-755 мышей

Препарат	Доза препаратов мг/кг	Срок введения препарата после прививки опухоли, дни	Торможение роста опухоли, % к контролю		Отношение числа погибших животных к числу животных в группе
			1 день	3 дня	
МТХ	2	8—12	12	+13	1/6
NSC-176319	5	8—12	12	+8	0/6
МТХ+NSC-176319	2+5 (вводились одновременно)	8—12	76	79	5/6

Таблица 6

Действие на рост Са-755 мышей МТХ, NSC-176319 и комплекса трихлорметилкобаламина с палладием

Препарат	Доза препаратов, мг/кг	Срок введения препаратов после прививки опухоли, дни	Торможение роста опухоли, % к контролю			Увеличение продолжительности жизни, % к контролю
			2 дня	8 дней	14 дней	
МТХ	10	2-й и 6-й	99	51	0	14
NSC-176319	5	2-й и 6-й	37	7	+29	0
$MetCbl \cdot PdCl_2$	250	2-й и 6-й	75	40	13	0
NSC-176319+$MetCbl \cdot PdCl_2$	5+250 (вводились одновременно)	2-й и 6-й	90	58	4	23
$MetCbl \cdot PdCl_2$+МТХ	250+10 (МТХ вводился через 20 мин после $MetCbl \cdot PdCl_2$)	2-й и 6-й	99	58	5	8
NSC-176319+МТХ	5+10 (МТХ вводился через 20 мин после NSC-176319)	2-й и 6-й	99	88	44	0
NSC-176319+$MetCbl \cdot PdCl_2$+МТХ	5+250+10 (NSC-176319 и $MetCbl \cdot PdCl_2$ вводились одновременно, а МТХ-через 20 мин после них)	2-й и 6-й	00	95	85	20

опухолей практически отсутствовало. Однако следует отметить, что одновременно повышалась токсичность. Установлено также, что действие комбинации препаратов существенно изменялось в зависимости от последовательности введения комбинантов и интервалов между ними. Так, одновременное введение NSC-176319 и МТХ оказалось значительно менее токсичным для организма, чем введение их с интервалом 3 ч при равном противоопухолевом эффекте.

Таким образом, результаты экспериментальных исследований подтверждают наше предположение о возможности усиления противоопухолевого действия МТХ с помощью аналогов метилкобаламина и ингибитора метионинсинтетазы. Это открывает новый подход к лечебному воздействию на опухоли с использованием антагонистов физиологического регулятора обмена соединений фолиевой кислоты в организме. Нами установлена противоопухолевая активность антагонистов кобаламинового кофермента. Однако активность исследованных аналогов метилкобаламина, блокирующих определенные метаболические звенья, недостаточна высока для полного и длительного торможения роста опухоли. Противоопухолевое действие аналогов кобаламинового кофермента может быть значительно усилено путем их комбинированного применения с МТХ. Полученные экспериментальные данные указывают на целесообразность испытания эффективности аналогичного рода комбинаций в клинике. Наша основная задача в настоящее время состоит в разработке оптимального режима комбинированного лечения опухолей указанными препаратами на основе всестороннего анализа механизма их сочетанного действия в организме.

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POSSIBILITY OF POTENTIATING THE ANTINEOPLASTIC ACTION OF FOLIC ACID ANTAGONIST BY METHYLCOBALAMINE ANALOGUES

Z. P. Sofyina, N. V. Myasischeva, F. G. Arsenyan, A. M. Yurkevich

Summary. The effect of methylcobalamine and its analogues (difluoro-chloromethylcobalamine — CF_2ClCbl and methylcobalamine chloropalladate — $MetCbl \cdot DdCl_2$) on the growth of transplantable tumours in mice: adenocarcinoma of the mammary gland (Ca-755), carcinoma of the uterine cervix (CUC-5), carcinoma of the intestine (ACATOL) was studied. The activity of the cobalamine coenzyme analogues was investigated when used alone or combined with inhibitors of dehydrofolate reductase and methyonine synthetase. The results of the experiments indicate a stimulating effect of methylcobalamine on the growth of transplantable solid tumours in the animal organism. The antitumour activity of the methylcobalamine analogues studied was found to be higher in combined application with methotrexate. The most effective inhibition of tumour growth and the longer survival of the animals were achieved in combined application of methylcobalamine with methotrexate and methyonine synthetase inhibitor, depending upon the scheme of administration.

СОДЕРЖАНИЕ

CONTENTS

РЕФЕРАТЫ СТАТЕЙ, ОПУБЛИКОВАННЫХ В ЭТОМ НОМЕРЕ

УДК 61:612.017.1

Иммунология в современной медицине. Косяков П. Н. Вестн. АМН СССР, 1979, № 1, с. 14.

Отмечается значение иммунологии для многих разделов современной медицины: прежде всего иммунологии инфекций, а также многих разделов неинфекционной иммунологии. Иммунологические методы благодаря их уникальной специфичности и высокой чувствительности нашли самое широкое применение в различных областях биологии и медицины. Указывается, что иммунные реакции, защитные по своей природе, в силу тех или других причин могут быть извращены и направлены не только на чужеродные антигены, но и на некоторые собственные, нормальные, неизменные антигены клеток и тканей, в результате чего возникают истинные аутоиммунные болезни.

Библиография: 15 названий.

УДК 612.017.1:001.8

Современные взгляды на пути развития иммунологии (проблемы и перспективы). Бароян О. В., Каулен Д. Р. Вестн. АМН СССР, 1979, № 1, с. 21.

Представлены основные задачи, стоящие перед иммунологией. Рассматривается главная задача — возможности поисков путей целенаправленной регуляции иммунного ответа организма. Авторы видят такую возможность в разработке способов, регулирующих клеточные кооперации, модификации клеточного микроокружения, использовании фрагментов антител. Обращается внимание на значение растворимых медиаторов клеточного иммунитета — лимфокинов. Особо отмечено влияние

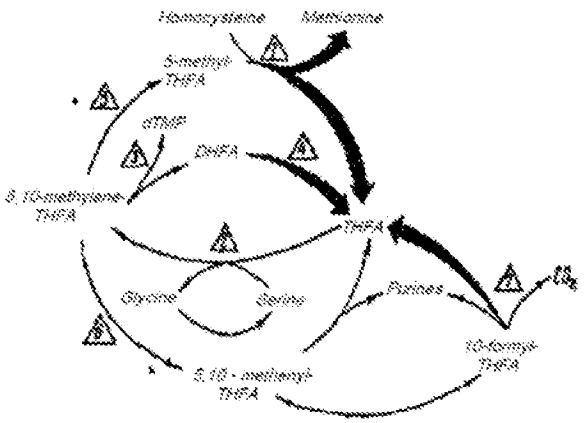
UDK 616-006-092.9:615.277.3

N.V. MYASISHCHEVA, Z.P. SOF'YINA,
C.D. GOLENKO, F.G. ARSENYAN

Antitumor efficiency of methotrexate in combination with cobalamine derivatives

Clinical and experimental studies have shown that one of the coenzymes of B-12 vitamin, methylcobalamine, under certain conditions demonstrates properties typical for modifying factors of cancerogenesis [1]. Specifically, carcinogenic activity of some metabolites of tryptophane and tyrosine, including para-oxy-phenyl-lactic acid, inside animals' bodies increases when introduced simultaneously with cobalamine coenzyme. The effect of methylcobalamine leads to considerable reduction in latent period during appearance of induced hemoblastoses and significantly increases the frequency of their development [2-4]. Methylcobalamine also stimulates the growth of transplantable tumors [5]. High biological activity of methylcobalamine is generally caused by its role as a coenzyme of methionine synthetase (K.F.2.1.1.13), which controls folate metabolism in mammalian cells [6, 7]. The concluding stage of methionine biosynthesis represents the main triggering mechanism of the folate-dependent reactions cycle in the process of cell growth during purine and pyrimidine formation [8] (Figure).

The normal balance between free tetrahydrofolic acid (THFA) and its single-carbon derivatives depends on the intensity of purine and pyrimidine synthesis, as well as activity of methionine synthetase and dehydrofolate reductase. As the concentration of methionine goes up, the activity of formyl-THFA dehydrogenase increases. This cellular link enables control of folate exchange as well as the exchange of irreplaceable amino acid itself. The latter is required for providing an adequate amount of methionine for synthesis of polyamines and proteins [8].



Cycle of folic acid and conjugated processes of biosynthesis of methionine, purines and pyrimidines:
1 – cobalamine-dependent methionine synthetase, 2 – serine-oxy-methyl transferase, 3 – thymidylate synthetase, 4 – dehydrofolate reductase, 5 – methylene-THFA reductase, 6 – methylene-THFA dehydrogenase, 7 – formyl-THFA dehydrogenase.

Methionine also controls the content of methyl-THFA by reverse inhibition of methylene-THFA reductase. The high level of methyl-THFA reduces activity of serine-oxy-methyl transferase, necessary for methylene-THFA formation as well as subsequent formation of pyrimidines along with thymidylate synthetase. Methylene-THFA dehydrogenase also assists with formation of 5, 10-methenyl- and formyl-THFA for purine synthesis. The transformation of the main transporting form of folic acid, methyl-THFA, into coenzymes (methylene- and formyl-THFA) is limited by cobalamine-dependent methionine synthetase. Deranged DNA synthesis in hematogenic human cells as a result of the "methyl-THFA trap", reduction in concentration of free THFA and its

coenzymes in case of B-12 vitamin deficiency confirm the role of methylcobalamine in this process [9, 10]. At the same time, its high blood serum content in patients with acute leucosis is likely one of the reasons of their low sensitivity to combined chemotherapy due to development of the cobalamine-dependent rescue path in tumor cells [11].

Our experimental data concerning modifying effect of cobalamines on the processes of growth of transplantable and induced tumors in the bodies of animals justified the real possibility of using cobalamine derivatives to increase efficiency of chemotherapy. In this study, main attention was paid to analysis of the effect of methylcobalamine and its antagonists on antitumor activity of methotrexate in case of transplantable solid tumors in animals.

Method of study. Experiments were conducted using mice of the following lines: $C_{57}B1/6$, BALB/c, F_1, BDF ($C_{57}B1_xDBA/2$), F_3 ($C_{57}B1_xCBA$), as well as SHK mice obtained from the nursery of the USSR Academy of Medical Sciences (AMN). We used the following models of transplantable solid tumors: adenocarcinoma of the mammary gland (Ca-755), cancer of the uterine cervix (CUC-5), adenocarcinoma of the large intestine (ACATOL), Lewis lung cancer (LLC) and sarcoma 37 (C-37). Chemotherapeutic tests were conducted according to the previously published scheme [5]. The effect of methylcobalamine was studied in different series by intramuscular introduction thereof and its two antagonists: diflouro-chloro-methylcobalamine and methylcobalamine chloropalladate on the 3rd and 7th day after transplantation of the tumors. The drugs were administered in the dose of 250 mg/kg of mass daily for 5 days, first – intramuscular, and second – perorally. Cobalamine derivatives were synthesized at the research and production facility "Vitaminy". We also used methotrexate manufactured by "Lederle" (USA). The resulting effects of the drugs on solid tumors were evaluated directly 24 hours after the course of treatment as well as in the long term. A percentage of stimulation and inhibition of tumor growth, calculated based on conditional volume ($(\frac{v_0 - v_k}{v_k} x100\%)$), as well as the increase in animals' life span were used as the efficiency criteria in this study. Proliferative activity of the tumor cells was studied using the autoradiography method with ^{3}H-thymidine.

Results and discussion. A stimulating effect of methylcobalamine on the growth of transplantable solid tumors has been established. Administration of low doses of methylcobalamine considerably increases the growth of Ca-755 and ACATOL. In case of CUC-5 and C-37, the effect of methylcobalamine was less pronounced and shorter lasting (Table 1.).

T a b l e 1. Methylcobalamine effect on growth of transplantable tumors in mice

Tumor	Drug dose, mg/kg	Tumor volume, % vs control	
		Number of days since administering a drug	
		1 – 2	7 – 8
Ca-755	0.01	180	65
ACATOL	0.01	126	37
CUC-5	0.01	47	0
C-37	0.50	57	0

N o t e. Methylcobalamine was administered on days 3 and 7 of tumor growth. Results are statistically significant ($P < 0.05$) vs. control.

In the minimal dose (0.01 mg/kg of mass) methylcobalamine caused insignificant increase in Ca-755 growth and on day 5 after its administration the volume of tumor exceeded the control only by 56%. When administering methylcobalamine twice (total dose of 0.02 mg/kg of mass), on day 7 of the tumor growth its volume increased by 2.3 – 2.8 times as compared to the control. During the following 2-3 weeks, the growth slowed down. As was shown earlier [12], the degree and duration of stimulating Ca-755 growth increased with an increase in the dose of administered methylcobalamine and depended

upon the mice line. Specificity of methylcobalamine effect on the tumor growth processes confirms the results of comparative evaluation of activity of two cobalamine coenzymes. According to our data, as opposed to methylcobalamine, administering the animals with a similar dose of 5-deoxyadenosylcabolamine (K.F.5.4.99.2) practically had no effect on Ca-755 growth. Stimulating effect of methylcobalamine on the tumor growth determined the practicality of using some of its antagonists for inhibiting cobalamine-dependent reactions in animals' bodies. We used difluoro-chloromethyl-cobalamine and methylcobalamine chloropalladate as potential antitumor compounds. These derivatives of methylcobalamine inhibited cobalamine coenzymes entrance to the precursor cells and synthesis thereof in the culture of blast-transformed human blood lymphocytes [13]. Among specified analogues of methylcobalamine, the strongest antitumor activity was demonstrated by methylcobalamine chloropalladate. Depending on the administration scheme, the drug inhibited the growth of Ca-755, RMSh-5 and LLC by 70-80%. Under similar administration regime, difluoro-chloromethylcobalamine did not demonstrate pronounced activity, which confirmed our data obtained during *in vitro* studies. This is likely caused by inhibition of biosynthesis of adenosylcobalamine in the tumor cells.

To ensure justified use of cobalamines in the combined tumor chemotherapy, it was important to study various aspects of their effect on the animals' bodies with transplantable tumors. For this purpose, we have conducted *in vivo* analysis of the main parameters of cellular proliferation, biosynthesis of cobalamine coenzymes as well as activity of cobalamine-dependent methionine synthetase in tumor cells using Ca-755 model, which is the most sensitive to these drugs. According to the earlier obtained data, upon introduction of cobalamine coenzyme, the generation time (T_g) and the separate periods thereof (t_{g_1}; t_{g_2}; $t_{g_1} + t_m$) in case of Ca-755 cells does not change and constitutes 12, 6, 2, and 4 hours respectively [8, 14]. As a result of methylcobalamine effect, the number of proliferating cells in the tumor increases significantly. It should be noted that the cell loss factor in the tumor is minimal and increases insignificantly under the effect of cobalamine coenzyme. Statistically significant variations in the index of ^{3}H-thymidine-labeled cells are seen in Ca-755 in mice 24 hour after administration of methylcobalamine. In the exponential tumor growth phase, under methylcobalamine effect the label index increases by 1.4 times as compared to control. We have obtained similar results upon multiple administration of ^{3}H-thymidine. The value of the label index in the tumor under the effect of methylcobalamine was significantly higher that that in the control group (56.9 ± 2.1% and 42.8 ± 1.3% respectively). The proliferative pool value, calculated based on comparison between the observed and expected label indices, has also increased considerably upon introduction of methylcobalamine.

It is know that in the process of growth of the majority of solid tumors in animals, the pool of proliferating cells decreases. In Ca-755, on days 6 – 14 of their growth, the number of labeled cells upon introduction of ^{3}H-thymidine gradually decreases from 28 to 9%. Under the effect of methylcobalamine in the process of Ca-755 growth, a decrease in the label index is seen as well. However, the increased number of labeled cells in the tumor remains at the later stages of growth as well. Hence, upon introduction of a small dose of methylcobalamine, a considerable increase is seen in the pool of proliferating cells, which are the most sensitive to inhibiting action of cyclo-specific substances. When studying the kinetics of growth and proliferation of Ca-755 cells, we have also evaluated the effect of

methylcobalamine chloropalladate. Upon introduction of the latter to animals, the inhibition of tumor growth is seen in the early exponential phase [15]. The indices of the pool of proliferating cells in the tumor 48 hours after introduction of methylcobalamine chloropalladate already demonstrate almost no difference as compared to their values in the control.

The combination of the obtained data allowed formulation of a conceptually new approach to the combined chemotherapy of tumors by utilizing the modifying effect of cobalamines on the processes of tumor growth. Considering synergism of the folic acid compounds and cobalamines activity in the processes of cell proliferation, it appeared possible to considerably increase the antitumor activity of methotrexate. We have studied alternative ways to increase the antitumor activity of methotrexate by using it in combination with cobalamine derivatives. By increasing the pool of proliferating cells in the tumor using methylcobalamine, it was possible to achieve increased selectivity of methotrexate. Considerable increase in antitumor activity of methotrexate was observed when it was used in combination with methylcobalamine in animals with Ca-755, CUC-5 and leucosis L 1210. The antitumor effect of methotrexate, which is a specific inhibitor of dehydrofolate reductase, also increases noticeably in case of simultaneous blocking of methionine synthetase in the tumor cells using methylcobalamine antagonist or Kein derivative of quinoline [16, 17]. It should be noted that antitumor activity of the studied methylcobalamine antagonists is insignificant and does not provide long-term inhibition of the tumor growth. However, limitation of the rescue path of folic coenzyme formation in the cells creates sufficient background for increasing antitumor effect of methotrexate (see Figure). When used in combination with methylcobalamine chloropalladate, the inhibition of growth of Ca-755, CUC-5 and ACATOL increases considerably and takes place when there is practically no activity from methotrexate by itself (Table 2).

T a b l e 2. Combined effect of methotrexate, cobalamine derivatives and Kein quinoline on growth of Ca-755 in C57Bl mice.

Drug	Drug dose, mg/kg	Inhibition of tumor growth, % vs. control			Increase in animals life span, % vs. control
		Number of days since administering a drug			
		1-2	7-8	14-15	
Methotrexate	10	75	44	18	0
Methotrexate + methylcobalamine	10+0.01	99	70	9*	19*
Methotrexate + methylcobalamine chloropalladate	10+250	98	88	–	0
Methotrexate + methylcobalamine chloropalladate + Kein quinoline	10+250 + 6	100	96	85	30

N o t e. Drugs were administered on day 3 of tumor growth; methotrexate and Kein quinoline – via intraperitoneal administration, twice, 96 hours apart; methylcobalamine chloropalladate – five times, 24 hours apart. * $p > 0.05$

An important factor in the mechanism of stimulating effect of methylcobalamine is its ability to induce activity of methionine synthetase in the tumor cells. This was demonstrated earlier in the culture of mammalian cells and human tumor cells. However, not all the types of tumor cells are capable of *in vitro* biosynthesis of methionine, which is required for their growth [18]. Therefore, it is worth mentioning our experimental data concerning increased activity of methionine synthetase in Ca-755 cells as a result of administering animals with methylcobalamine [15]. It is obvious that the decrease in the amount of holoenzyme as a result of methylcobalamine chloropalladate effect is caused by reduced supply of the active forms of cobalamines to the tumor cells as a result of the competition for transporting protein, transcobalamine II. Our experimental

data confirm that characteristic accumulation of cobalamine cyan-Co^{57} by the tumor in the exponential phase of Ca-755 growth, as well as formation of labeled cobalamine coenzymes in the cells decrease rapidly upon administering animals with methylcobalamine antagonist [19].

We believe that enhancement of antitumor activity of methotrexate when used in combination with methylcobalamine largely depends on the possibility of inhibition of DNA synthesis in the majority of tumor cell population. And, although the effect of methylcobalamine on the rate of methotrexate delivery to the tumor cells is not excluded, clarification of this aspect requires special studies. Currently, we are in possession of a new experimental evidence of methylcobalamine-assisted increase in antitumor activity of yet another S-phase specific antimetabolite – arabinosylcytosine.

N.V. Myasishcheva, Z.P. Sof'yina, C.D. Golenko, F.G. Arsenyan

ANTITUMOR EFFICIENCY OF METHOTREXATE IN COMBINATION WITH COBALAMINE DERIVATIVES

Summary

Improvement of the antitumor activity of methotrexate is suggested on the basis of modifying action of cobalamine on processes of the tumor growth. The influence of methylcobalamine and two of its analogues is shown on various experimental models: Ca-755, Ca cervix uteri and colon, LLC, sarcoma 37 of mice.

Two alternative ways to increase the therapeutic efficiency of methotrexate are analyzed with due regard for kinetic parameters of cell proliferation, of Cbl-coenzymes biosynthesis and activity of Cbl-dependent methionine synthetase in tumor.

All-Union Cancer Research Center,
Academy of Medical Sciences, USSR, Moscow

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All-Union Cancer Research Center,
Academy of Medical Sciences, USSR, Moscow

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Всесоюзный онкологический научный центр АМН СССР, Москва

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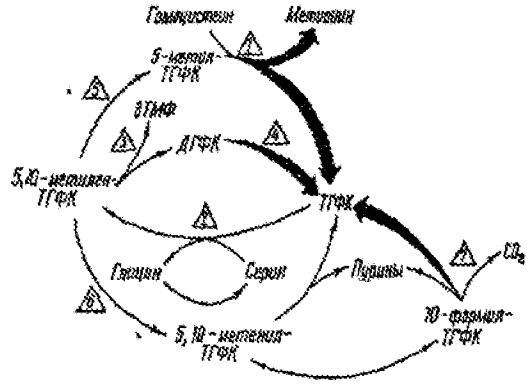
Противоопухолевая эффективность метотрексата при его комбинированном применении с кобаламиновыми производными

Н. В. МЯСИЩЕВА, З. П. СОФЬИНА, О. Д. ГОЛЕНКО, Ф. Г. АРСЕНЯН

Клинико-экспериментальные исследования показали, что один из коферментов витамина B_{12}— метилкобаламин в определенных условиях проявляет свойства, характерные для модифицирующих факторов канцерогенеза [1]. В частности, канцерогенная активность некоторых метаболитов триптофана и тирозина, включая пара-оксифенилмолочную кислоту, в организме животных возрастает при одновременном введении с кобаламиновым коферментом. Воздействие метилкобаламина приводит к значительному сокращению латентного периода при возникновении индуцированных гемобластозов и существенно повышает частоту их развития [2—4]. Метилкобаламин оказывает также стимулирующее действие на рост перевиваемых опухолей [5]. Высокая биологическая активность метилкобаламина в основном обусловлена его ролью кофермента метионинсинтетазы (К.Ф.2.1.1.13), контролирующей в клетках млекопитающих метаболизм фолата [6, 7]. Завершающий этап биосинтеза метионина составляет главный пусковой механизм цикла фолатзависимых реакций в процессе роста клеток при образовании пуринов и пиримидинов [8] (рисунок).

Нормальный баланс между свободной тетрагидрофолиевой кислотой (ТГФК) и ее одноуглеродистыми производными зависит от интенсивности синтеза пуринов и пиримидинов, а также активности метионинсинтетазы и дигидрофолатредуктазы. При увеличении концентрации метионина возрастает активность формил-ТГФК дигидрогеназы. С помощью этого звена в клетках регулируется не только обмен фолатов, но и самой незаменимой аминокислоты. Последнее необходимо для предоставления адекватного количества метионина для синтеза полиаминов и белка [8]. Путем обратного торможения метилен-ТГФК редуктазы метионин контролирует также содержание метил-ТГФК. Высокий уровень последней сни-



Цикл фолиевой кислоты и сопряженных процессов биосинтеза метионина, пуринов и пиримидинов:

1 — кобаламинзависимая метионинсинтетаза, *2* — серингидроксиметилтрансфераза, *3* — тимидилатсинтетаза, *4* — дигидрофолатредуктаза, *5* — метилен-ТГФК редуктаза, *6* — метилен-ТГФК дегидрогеназа, *7* — формил-ТГФК дегидрогеназа.

жает активность серингидроксиметилтрансферазы, необходимой для образования метилен-ТГФК и последующего формирования пиримидинов совместно с тимидилатсинтетазой. При участии метилен-ТГФК дегидрогеназы осуществляется также образование 5, 10-метенил- и формил-ТГФК для синтеза пуринов. Превращение основной транспортной формы фолиевой кислоты — метил-ТГФК в коферменты (метилен- и формил-ТГФК) лимитирует кобаламинзависимая метионинсинтетаза. Нарушение синтеза ДНК в кроветворных клетках человека в результате «ловушки метил-ТГФК», снижения концентрации свободной ТГФК и ее коферментов при недостаточности витамина B_{12} подтверждает значимость в этом процессе метилкобаламина [9, 10]. Вместе с тем его высокое содержание в сыворотке крови больных острым лейкозом является, по-видимому, одной из причин их малой чувствительности к комбинированной химиотерапии благодаря развитию кобаламинзависимого спасательного пути в опухолевых клетках [11].

Наши экспериментальные данные о модифицирующем действии кобаламинов на процессы роста перевиваемых и индуцируемых опухолей в организме животных обосновали реальную возможность использования кобаламиновых производных для повышения эффективности химиотерапии. В данных исследованиях основное внимание уделяли анализу действия метилкобаламина и его антагонистов на противоопухолевую активность метотрексата при перевиваемых солидных опухолях животных.

Методика исследований. Опыты проведены на 420 мышах линии C57Bl/6, BALB/c, F_1, BDF (C57Bl×ДВА/2), F_2 (C57Bl×CBA), а также на мышах SHK, полученных из питомника АМН СССР. Нами использованы следующие модели перевиваемых солидных опухолей: аденокарцинома молочной железы (Ca-755), рак шейки матки (РШМ-5), аденокарцинома толстого кишечника (АКАТОЛ), рак легкого Льюис (LLC) и саркома 37 (С-37). Химиотерапевтические опыты проведены в соответствии с ранее опубликованной схемой [5]. В разных сериях исследовано влияние метилкобаламина, который вводили внутримышечно на 3-и и 7-е сутки после перевивки опухолей и двух его антагонистов: хлордифтор- и хлорпалладата метилкобаламина. Препараты вводили в дозе 250 мг/кг массы ежедневно в течение 5 суток, первый — внутримышечно, а второй — перорально. Кобаламиновые производные были синтезированы в научно-производственном объединении «Витамины». Использовали также метотрексат («Lederle», США). Результаты действия препаратов на солидные опухоли оценивали непосредственно через 24 ч после курса лечения и в отдаленные сроки. Критерием эффективности при этом служили процент стимуляции и торможения роста опухоли, вычисляемые по условному объему $\left(\frac{v_a - v_k}{v_k} 100\ \%\right)$, и увеличение продолжительности жизни животных. Пролиферативную активность опухолевых клеток исследовали с помощью метода авторадиографии с ^3H-тимидином.

Результаты исследований и их обсуждение. Установлено стимулирующее действие метилкобаламина на рост перевиваемых солидных опухолей. Введение малых доз метилкобаламина значительно ускоряет рост Ca-755 и АКАТОЛ. Не столь выраженное и более кратковременное стимулирующее действие метилкобаламина выявлено при РШМ-5 и С-37 (табл. 1). В минимальной дозе (0,01 мг/кг массы) метилкобаламин усиливал рост Ca-755 незначительно и на 5-е сутки после его введения объем опухоли превышал контроль лишь на 56 %. При двукратном введении метилкобаламина (суммарная доза 0,02 мг/кг) на 7-е сутки роста опухоли объем ее увеличивался в 2,3—2,8 раза по сравнению с контролем. В последующие 2—3 недели рост опухоли замедлялся. Как показано ранее [12], степень и продолжительность стимуляции роста Ca-755 возрастали с увеличением дозы вводимого метилкобаламина и зависели от линии мышей. Специфичность действия метилкобаламина на процессы роста опухоли подтверждают результаты сравнительной оценки активности двух кобаламиновых коферментов. Согласно нашим данным, в отличие от метилкобаламина введение животным аналогичной дозы 5-дезоксиаденозилкобаламина (К. Ф. 5. 4. 99. 2) практически не влияло на

Таблица 1. Влияние метилкобаламина на рост перевиваемых опухолей мышей

Опухоль	Доза препарата, мг/кг	Объем опухоли, % к контролю	
		Сроки после окончания введения препарата, сутки	
		1—2	7—8
Ca-755	0,01	180	66
АКАТОЛ	0,01	126	37
РШМ-5	0,01	47	0
С-37	0,50	57	0

Примечание. Метилкобаламин вводили на 3-и и 7-е сутки роста опухоли. Результаты статистически достоверны ($P<0,05$) по отношению к контролю.

рост Са-755. Стимулирующее действие метилкобаламина на рост опухолей определило целесообразность использования некоторых его антагонистов для торможения кобаламинзависимых реакций в организме животных. В качестве потенциальных противоопухолевых соединений нами были исследованы хлордифторметилкобаламин и хлорпалладат метилкобаламина. Эти производные метилкобаламина тормозили в культуре бласттрансформированных лимфоцитов крови человека поступление в клетки предшественника кобаламиновых коферментов и их биосинтез [13]. Из указанных аналогов метилкобаламина бо́льшую противоопухолевую активность проявил хлорпалладат метилкобаламина. В зависимости от схемы введения препарат тормозил рост Са-755, РМШ-5 и LLC на 70—80 %. При аналогичном режиме введения хлордифторметилкобаламин не проявил выраженной активности, что подтверждало наши данные, полученные при исследовании in vitro. По-видимому, это обусловлено происходящим торможением биосинтеза аденозилкобаламина в опухолевых клетках.

Для обоснованного применения кобаламинов в комбинированной химиотерапии опухолей важное значение имело изучение различных аспектов их действия на организм животных с перевиваемыми опухолями. С этой целью на модели Са-755, наиболее чувствительной к их воздействию, мы исследовали in vivo основные параметры пролиферации клеток, биосинтез кобаламиновых коферментов и активность кобаламинзависимой метионинсинтетазы в опухолевых клетках. Согласно полученным ранее данным, при введении кобаламинового кофермента время генерации (T_c) и его отдельных периодов (t_s; t_{g2}; $t_{g1}+t_m$) клеток Са-755 не изменяется и составляет соответственно 12, 6, 2, 4 ч [8, 14]. При воздействии метилкобаламина в опухоли существенно увеличивается количество пролиферирующих клеток. Следует отметить, что фактор потери клеток в опухоли минимален и возрастает несущественно при воздействии кобаламинового кофермента. Статистически значимые различия индекса меченных ^{3}H-тимидином клеток отмечаются в Са-755 мышей через 24 ч после введения метилкобаламина. В экспоненциальной фазе роста опухоли при воздействии метилкобаламина индекс метки увеличивается в 1,4 раза по сравнению с контролем. Аналогичные результаты получены нами при многократном введении ^{3}H-тимидина. Величина индекса метки в опухоли при воздействии метилкобаламина была существенно выше контроля (56,9±2,1 % и 42,8±1,3 % соответственно). Значение пролиферативного пула, рассчитанное методом сравнения наблюдаемого и ожидаемого индексов метки, также было значительно повышено при введении метилкобаламина.

Известно, что в процессе роста большинства со́лидных опухолей животных пул пролиферирующих клеток уменьшается. В Са-755 на 6—14-е сутки роста количество меченых клеток после однократного введения ^{3}H-тимидина постепенно снижается с 28 до 9 %. При воздействии метилкобаламина в процессе роста Са-755 также наблюдается уменьшение индекса метки. Однако при этом повышенное количество меченых клеток в опухоли сохраняется и в поздних стадиях роста. Таким образом, при введении небольшой дозы метилкобаламина наблюдается существенное увеличение пула пролиферирующих клеток, наиболее чувствительных к ингибирующему действию циклоспецифических веществ. При исследованиях кинетики роста и пролиферации клеток Са-755 мы оценивали также действие хлорпалладата метилкобаламина. При его введении животным торможение роста опухолей отмечается в ранней экспоненциальной фазе. [15]. Показатели пула пролиферирующих клеток в опухоли через 48 ч после воздействия хлорпалладата метилкобаламина уже существенно не отличаются от их значений в контроле.

Совокупность полученных нами данных позволила сформулировать принципиально новый подход к комбинированной химиотерапии опухолей на основе использования модифицирующего влияния кобаламинов на процессы их роста. Учитывая синергизм действия соединений фолиевой кислоты и кобаламинов в процессах пролиферации клеток, представлялось возможным существенно повысить противоопухолевую активность метотрексата. Мы исследовали альтернативные пути возрастания противоопухолевой активности метотрексата при его комбинированном применении с кобаламиновыми производными. Повышения избирательности действия метотрексата удалось достигнуть в результате увеличения в опухоли пула пролиферирующих клеток с помощью метилкобаламина. Значительное увеличение противоопухолевой активности метотрексата наблюдалось при его сочетанном применении с метилкобаламином у животных с Са-755, РШМ-5 и лейкозом L 1210. Противоопухолевый эффект метотрексата — специфического ингибитора дигидрофолатредуктазы заметно возрастает также при одновременном блокировании в опухолевых клетках метионинсинтетазы с помощью антагониста метилкобаламина или производного хинолина Кейна [16, 17]. Следует отметить, что противоопухолевая активность исследованных антагонистов метилкобаламина незначительна и не обеспечивает длительного торможения роста опухоли. Однако ограничение в клетках спасательного пути образования фолиевых коферментов создает достаточный фон для увеличения противоопухолевого действия метотрексата (см. рисунок). При его комбинированном

Таблица 2. Комбинированное действие метотрексата, кобаламиновых производных и хиколина Кейна на рост Са-755 мышей линии С57Bl

Препарат	Доза препарата, мг/кг	Торможение роста опухоли, % к контролю			Увеличение продолжительности жизни животных, % к контролю
		Сроки после введения препарата, сутки			
		1—2	7—8	14—15	
Метотрексат	10	75	44	18	0
Метотрексат+ +метилкобаламин	10+0,01	99	70	9*	19*
Метотрексат+ +хлорпалладат метилкобаламина	10+250	98	88	—	0
Метотрексат+ +хлорпалладат метилкобаламина+ +хиколин Кейна	10+ +250+5	100	96	85	30

Примечания. Препараты вводили на 3-и сутки роста опухоли; метотрексат и хиколин Кейна — внутрибрюшинно двукратно с интервалом 96 ч; хлорпалладат метилкобаламина — пятикратно с интервалом 24 ч. * $p > 0,05$

применении с хлорпалладатом метилкобаламина торможение роста Са-755, РШМ-5, АКАТОЛ существенно возрастает и проявляется в сроки, когда активность одного метотрексата практически отсутствует (табл. 2).

В механизме стимулирующего действия метилкобаламина важным является его способность индуцировать активность метионинсинтетазы в опухолевых клетках. Это продемонстрировано ранее в культуре клеток млекопитающих и опухолевых клетках человека. Однако не все виды опухолевых клеток могут осуществлять in vitro биосинтез метионина, необходимый для их роста [18]. В связи с этим заслуживают внимания наши экспериментальные данные о возрастании активности метионинсинтетазы в клетках Са-755 при введении животным метилкобаламина [15]. Очевидно, снижение количества холофермента при воздействии хлорпалладата метилкобаламина обусловлено меньшим поступлением в опухолевые клетки активных форм кобаламинов в результате конкуренции за транспортный белок — транскобаламин II. Наши экспериментальные данные подтверждают, что характерное накопление опухолью цианкобаламина в экспоненциальной фазе роста Са-755 и формирование в клетках меченых кобаламиновых коферментов резко снижаются при введении животным антагониста метилкобаламина [19].

Усиление противоопухолевой активности метотрексата при одновременном применении с метилкобаламином, как мы полагаем, в значительной степени зависит от возможности торможения синтеза ДНК в большей части популяции опухолевых клеток. И хотя не исключено влияние метилкобаламина на скорость поступления метотрексата в опухолевые клетки [17], выяснение этого вопроса требует специальных исследований. В настоящее время мы располагаем новыми экспериментальными доказательствами повышения с помощью метилкобаламина противоопухолевой активности и другого S-фазовоспецифичного антиметаболита — арабинозидцитозина.

M. V. Myasishcheva, Z. P. Sofina, O. D. Golenko, F. G. Arsenyan

ANTITUMOUR EFFICIENCY OF METHOTREXATE IN COMBINATION WITH COBALAMINE DERIVATIVES

Summary

Improvement of the antitumour activity of methotrexate is suggested on the basis of modifying action of cobalamine on processes of the tumour growth. The influence of methylcobalamine and two of its analogues is shown on various experimental models: Ca—755, Ca cervix uteri and colon, LLC, sarcoma 37 of mice.

Two alternative ways to increase the therapeutic efficiency of methotrexate are analyzed with due regard for kinetic parameters of cell proliferation, of Cbl-coenzymes biosynthesis and activity of Cbl-dependent methionine synthetase in the tumour.

All-Union Cancer Research Centre,
Academy of Medical Sciences, USSR, Moscow

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Всесоюзный онкологический научный центр АМН СССР, Москва

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Молекулярные механизмы регуляции роста опухолей молочной железы эстрогенами

Т. М. МОРОЗОВА, Т. И. МЕРКУЛОВА, Р. И. САЛГАНИК

Известно, что эстрогены в тканях-мишенях (матке, молочной железе) активируют пролиферацию эпителиальных тканей; этому предшествует индукция синтеза РНК и белков-ферментов, обеспечивающих последующий синтез ДНК и митозы. Нередко под контролем эстрогенов находится также и рост опухолей молочной железы (ОМЖ) экспериментальных животных и человека. В таких опухолях, как и в нормальных тканях, эстрадиол стимулирует синтез РНК, белков, ДНК и активирует клеточное деление. Однако часть опухолей в ходе малигнизации теряет способность реагировать на эстрадиол; такие опухоли становятся эстрадиолнезависимыми.

Изучение молекулярных механизмов взаимодействия эстрадиола с клетками опухолей позволяет выяснить причины утраты зависимости роста опухолей от эстрогенов, что имеет не только теоретическое значение, так как для определения правильной тактики лечения больных с ОМЖ и для обоснованного применения эндокринной терапии необходимо установить, зависит ли рост данной опухоли от эстрогенов. В настоящее время нет достаточно надежных способов дискриминации эстрогензависимых и эстрогеннезависимых ОМЖ. Создание таких способов возможно на основании знания молекулярных механизмов действия эстрадиола на опухолевые клетки. Нашей задачей было изучение взаимодействия эстрадиола с эстрогензависимыми и эстрогеннезависимыми опухолями, выяснение механизмов нарушений в цепи для того, чтобы на этой основе разработать критерии оценки эстрогензависимости опухолей.

Опыты проводили на ОМЖ мышей высокораковых линий DD и C3H и на индуцированных 7,12-диметилбензантраценом (ДМБА) ОМЖ крыс линии Sprague-Dawley. Об эстрогензависимости опухолей судили по влиянию на их рост овариоэктомии животных и введения им эстрадиола [1]. Эстрогензависимыми считали опухоли, которые регрессировали после овариоэктомии. Введение эстрадиола стимулировало рост этих опухолей. Были выделены эстрогеннезависимые опухоли, на рост которых не влияли ни овариоэктомия, ни введение эстрадиола. Для выяснения причин утраты зависимости роста опухолей от эстрогенов прежде всего исследовали содержание рецепторов эстрадиола в клетках эстрогензависимых и эстрогеннезависимых опухолей (рис. 1), которое определяли по опи-

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11776329
	Filing Date	2007-07-11
	First Named Inventor	Clet NIYIKIZA
	Art Unit	1614
	Examiner Name	Kevin E. Weddington
	Attorney Docket Number	X14173B_US

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Signature	/Elizabeth A. McGraw/	Date (YYYY-MM-DD)	2009-12-15
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Application Number:	11776329
International Application Number:	
Confirmation Number:	6568
Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Customer Number:	25885
Filer:	Elizabeth Ann McGraw/Linda Durbin
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	Examiner Name	Kevin E. Weddington
	Attorney Docket Number	X14173B_US

	1	Maysishecheva, N.V., et al.: "Antitumor Activity of Methotrexate When Used in Combination with Cobalamine Derivatives", Eksperimentalnaya Onkologija (1982), vol. 4, no. 5:29-33.	☐
	2	McDonald, A.C., et al.: "Clinical Phase I Study of LY231514, a Multitargeted Antifolate, Administered by Daily x 5 q 21 Schedule", Annals of Oncology (1996), vol. 7:85, Abstract No. 291.	☐
	3	Sofyina, Z.P., et al.: "Possibility of Potentiating the Antineoplastic Action of Folic Acid Antagonist by Methylcobalamine Analogs", Vestnik Akademii Medicinskich Nauk SSSR (1979), vol. 1: 72-78.	☐

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/776,329	07/11/2007	Clet Niyikiza	X14173B	6568

25885 7590 02/05/2010

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

EXAMINER
WEDDINGTON, KEVIN E

ART UNIT	PAPER NUMBER
1614	

NOTIFICATION DATE	DELIVERY MODE
02/05/2010	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patents@lilly.com

Office Action Summary	Application No.	Applicant(s)	
	11/776,329	NIYIKIZA ET AL.	
	Examiner	Art Unit	
	KEVIN WEDDINGTON	1614	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *13 November 2009*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *40-44 and 47-63* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *40-44 and 47-63* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *11-13-09; 12-15-09*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

Claims 40-44 and 47-63 are presented for examination.

Applicants' amendment, response and information disclosure statement filed November 13, 2009; and the information disclosure statement filed December 15, 2009 have been received and entered.

Accordingly, the rejection made under 35 USC 103(a) as being obvious over Taylor (5,344,932) of PTO-1449 in view of Tsao et al., Pathobiology, vol. 61, No. 2, pp. 104-108 (1993) of PTO-1449, further in view of Worzalla et al., Anticancer Research, Vol. 18, No. 5, pp. 3255-3239 of PTO-1449, and further in view of Cleare et al. (4,149,707) as set forth in the Office action dated September 8, 2009 at pages 2-5 as applied to claims 40-52 is hereby withdrawn because of applicants' remarks.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 40-44 and 47-63 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-7 of U.S. Patent No. 7,053,065 B2. Although the conflicting claims are not identical, they are not patentably distinct from each other because the only difference between the present claims and the patented claims lies in that in the present claims, addition agent(s) is administered with the presently claimed active agents (pemetrexed disodium and vitamin B12).

The present claims would anticipate the patented claims because the patented claims recite "**comprising**" and thus opens the claims to the inclusion of additional active agent(s).

Claims 40-44 and 47-63 are not allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to KEVIN WEDDINGTON whose telephone number is (571)272-0587. The examiner can normally be reached on 12:30 pm - 9:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ardin Marschel can be reached on (571)272-0718. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

KEVIN WEDDINGTON
Primary Examiner
Art Unit 1614

/KEVIN WEDDINGTON/
Primary Examiner, Art Unit 1614

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11776329	NIYIKIZA ET AL.
	Examiner	**Art Unit**
	Kevin E Weddington	1614

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	02/11/2009	09/01/2009	01/28/2010						
	1			-						
	2			-						
	3			-						
	4			-						
	5			-						
	6			-						
	7			-						
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	35			-						
	36			-						

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CLAIM		DATE								
Final	Original	02/11/2009	09/01/2009	01/28/2010						
	37			-						
	38			-						
	39			-						
	40	✓	✓	✓						
	41	✓	✓	✓						
	42	✓	✓	✓						
	43	✓	✓	✓						
	44	✓	✓	✓						
	45	✓	✓	-						
	46	✓	✓	-						
	47	✓	✓	✓						
	48	✓	✓	✓						
	49	✓	✓	✓						
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	61			✓						
	62			✓						
	63			✓						

PTO/SB/08a (07-09)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	11776329
Filing Date	2007-07-11
First Named Inventor	Clet Niyikiza
Art Unit	1614
Examiner Name	
Attorney Docket Number	X14173B

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	1							☐

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	2007-07-11
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	Art Unit	1614
	Examiner Name	
	Attorney Docket Number	X14173B

/K.W./	1	ALIMTA, NDA 021462, Approved Label of 07/02/2009.	☐
	2	"Clinical Chemistry: principle, procedures, correlations," 3rd edition, 1996, published by Lippincott: pp. 618-627.	☐
	3	Fluorouracil, Physicians Desk References, (c) 1998, pp 2463-2464.	☐
	4	HAMMOND, L., et al., "A phase I and pharmacokinetic (PK) study of the multitarget antifol (MTA) LY231514 with folic acid, " American Society of Clinical Oncology (ASCO) Meeting Abstract No. 866 (1998).	☐
	5	KISLIUK, RL., 1984. "The Biochemistry of Folates." In Sirotnak (Ed.), Folate Antagonists as Therapeutic Agents. pp. 2-68. Harcourt Brace Jovanovich, Publishers.	☐
	6	KISLIUK, RL., 1999. "Folate Biochemistry in RElation to Antifolate Selectivity." In Jackson (Ed.), Antifolate Drugs in Cancer Therapy. pp 13-36. Humana Press, New Jersey.	☐
	7	Leucovorin, Physicians Desk Reference, (c) 1999. pp 1389-1391.	☐
	8	Methotrexate, Physicians Desk Reference, (c) 1999. pp. 1397-1413.	☐
	9	MORGAN, et al., "Folic acid supplementation prevent deficient blood folate levels and hyperhomocysteinemia during long-term, low dose methotrexate therapy for rheumatoid arthritis: implications for cardiovascular disease prevent," J. Rheumatol. 25:441-446. (1998).	☐
	10	NIYIKIZA, C., et al., "LY231514 (MTA): relationship of vitamin metabolite profile to toxicity," American Society of Clinical Oncology (ASCO) Meeting Abstract No. 2139 (1998).	☐
/K.W./	11	Raltitrexed, The Complete Drug Reference, Martindale, 32nd Ed., Pharmaceutical Press, London, pp 560.	☐

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/K.W./	12	SHIH, C., et al., "LY231514, a Pyrrolo[2,3-d]pyrimidine-based Antifolate that Inhibits Multiple Folate-requiring Enzymes," Cancer Research. 57:1116-1123. 1997.	☐
/K.W./	13	SHIH, C., et al., "Preclinical Pharmacology Studies and the Clinical Development of a Novel Multitargeted Antifolate, MTA (LY231514)," In Jackson (Ed.), Antifolate Drugs in Cancer Therapy. pp 13-36. Humana Press, New Jersey.	☐
/K.W./	14	VOLKOV, I., "The master key effect of vitamin B12 in treatment of malignancy - A potential therapy?", Medical Hypotheses. 70:324-328. 2008.	☐
	15		☐

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EXAMINER SIGNATURE

Examiner Signature	/Kevin Weddington/	Date Considered	01/25/2010

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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First Named Inventor	Clet NIYIKIZA
Art Unit	1614
Examiner Name	Kevin E. Weddington
Attorney Docket Number	X14173B_US

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

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	First Named Inventor	Clet NIYIKIZA
	Art Unit	1614
	Examiner Name	Kevin E. Weddington
	Attorney Docket Number	X14173B_US

/K.W./	1	Maysishecheva, N.V., et al.: "Antitumor Activity of Methotrexate When Used in Combination with Cobalamine Derivatives", Eksperimentalnaya Onkologija (1982), vol. 4, no. 5:29-33.	☐
/K.W./	2	McDonald, A.C., et al.: "Clinical Phase I Study of LY231514, a Multitargeted Antifolate, Administered by Daily x 5 q 21 Schedule", Annals of Oncology (1996), vol. 7:85, Abstract No. 291.	☐
/K.W./	3	Sofyina, Z.P., et al.: "Possibility of Potentiating the Antineoplastic Action of Folic Acid Antagonist by Methylcobalamine Analogs", Vestnik Akademii Medicinskich Nauk SSSR (1979), vol. 1: 72-78.	☐

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EXAMINER SIGNATURE

Examiner Signature	/Kevin Weddington/	Date Considered	01/25/2010

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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NOTICE OF ALLOWANCE AND FEE(S) DUE

25885 7590 03/10/2010

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

EXAMINER	
WEDDINGTON, KEVIN E	
ART UNIT	PAPER NUMBER
1614	

DATE MAILED: 03/10/2010

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/776,329	07/11/2007	Clet Niyikiza	X14173B	6568

TITLE OF INVENTION: NOVEL ANTIFOLATE COMBINATION THERAPIES

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1510	$300	$0	$1810	06/10/2010

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PTOL-85 (Rev. 08/07) Approved for use through 08/31/2010.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

25885 7590 03/10/2010

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)

(Signature)

(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/776,329	07/11/2007	Clet Niyikiza	X14173B	6568

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nonprovisional	NO	$1510	$300	$0	$1810	06/10/2010

EXAMINER	ART UNIT	CLASS-SUBCLASS
WEDDINGTON, KEVIN E	1614	514-052000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:

❑ Issue Fee

❑ Publication Fee (No small entity discount permitted)

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

❑ A check is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

❑ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

❑ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/776,329	07/11/2007	Clet Niyikiza	X14173B	6568

25885 7590 03/10/2010

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

EXAMINER	
WEDDINGTON, KEVIN E	
ART UNIT	PAPER NUMBER
1614	

DATE MAILED: 03/10/2010

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 132 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 132 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

Notice of Allowability	Application No.	Applicant(s)	
	11/776,329	NIYIKIZA ET AL.	
	Examiner	Art Unit	
	KEVIN WEDDINGTON	1614	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *February 23, 2010*.

2. ☒ The allowed claim(s) is/are *40-44 and 47-63; renumbered 1-22*.

3. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☐ All b) ☐ Some* c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

4. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date See Continuation Sheet
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☒ Interview Summary (PTO-413), Paper No./Mail Date *2-23-2010* .
7. ☐ Examiner's Amendment/Comment
8. ☐ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

/KEVIN WEDDINGTON/
Primary Examiner
Art Unit: 1614

Continuation of Attachment(s) 3. Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date: 11-13-2009; 12-15-2009.

Interview Summary	Application No.	Applicant(s)	
	11/776,329	NIYIKIZA ET AL.	
	Examiner	Art Unit	
	KEVIN WEDDINGTON	1614	

All participants (applicant, applicant's representative, PTO personnel):

(1) *KEVIN WEDDINGTON*. (3)_____.

(2) *Elizabeth A. McGraw*. (4)_____.

Date of Interview: *23 February 2010*.

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☒ No.
If Yes, brief description: _____.

Claim(s) discussed: *The claims in general*.

Identification of prior art discussed: *Niyikiza et al. (7,053,065 B2)*.

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☒ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *The attorney of record, Ms. McGraw, stated that the Niyikiz et al. (7,053,065 B2) cannot be used in an Obviousness-Type Double Patenting rejection because the present application is a Divisional of Niyikiza et al. (7,053,065 B2) which has a restriction requirement. The Examiner agreeds that an ODP rejection should not had been made*.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

/KEVIN WEDDINGTON/ Primary Examiner, Art Unit 1614	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

———

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11776329	NIYIKIZA ET AL.
	Examiner	Art Unit
	Kevin E Weddington	1614

SEARCHED

Class	Subclass	Date	Examiner
514	52	2/11/09	KEW
514	77	2/11/09	KEW
514	249	2/11/09	KEW
514	251	2/11/09	KEW
514	265.1	2/11/09	KEW

SEARCH NOTES

Search Notes	Date	Examiner
Consultation with parent applications, 10/297,821 and 11/288,807	2/11/09	KEW
EAST and PALM for Inventors' Names	2/11/09	KEW
CAS-ONLINE search with MEDLINE, CA and USPATALL	9/1/2009	KEW
Updated Searches	2/23/2010	KEW

INTERFERENCE SEARCH

Class	Subclass	Date	Examiner
514	52	2/23/2010	KEW
514	77	2/23/2010	KEW
514	249	2/23/2010	KEW
514	251	2/23/2010	KEW
514	265.1	2/23/2010	KEW

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Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
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BIB DATA SHEET

CONFIRMATION NO. 6568

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/776,329	07/11/2007	510	1614	X14173B

APPLICANTS

Clet Niyikiza, Indianapolis, IN;
Paolo Paoletti, Indianapolis, IN;
James Jacob Rusthoven, Ancaster, CANADA;

**** CONTINUING DATA ***************************

This application is a DIV of 11/288,807 11/29/2005 ABN
which is a DIV of 10/297,821 12/05/2002 PAT 7,053,065
which is a 371 of PCT/US01/14860 06/15/2001
which claims benefit of 60/215,310 06/30/2000
and claims benefit of 60/235,859 09/27/2000 ABN
and claims benefit of 60/284,448 04/18/2001

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

08/31/2007

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☐ No Verified and Acknowledged /KEVIN E WEDDINGTON/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		IN	0	11	2

ADDRESS

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288
UNITED STATES

TITLE

NOVEL ANTIFOLATE COMBINATION THERAPIES

FILING FEE RECEIVED		
1546	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

PTO/SB/08a (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	11776329
Filing Date	2007-07-11
First Named Inventor	Clet NIYIKIZA
Art Unit	1614
Examiner Name	Kevin E. Weddington
Attorney Docket Number	X14173B_US

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							☐

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NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11776329
	Filing Date	2007-07-11
	First Named Inventor	Clet NIYIKIZA
	Art Unit	1614
	Examiner Name	Kevin E. Weddington
	Attorney Docket Number	X14173B_US

/K.W./	1	Maysishecheva, N.V., et al.: "Antitumor Activity of Methotrexate When Used in Combination with Cobalamine Derivatives", Eksperimentalnaya Onkologija (1982), vol. 4, no. 5:29-33.	☐
/K.W./	2	McDonald, A.C., et al.: "Clinical Phase I Study of LY231514, a Multitargeted Antifolate, Administered by Daily x 5 q 21 Schedule", Annals of Oncology (1996), vol. 7:85, Abstract No. 291.	☐
/K.W./	3	Sofyina, Z.P., et al.: "Possibility of Potentiating the Antineoplastic Action of Folic Acid Antagonist by Methylcobalamine Analogs", Vestnik Akademii Medicinskich Nauk SSSR (1979), vol. 1: 72-78.	☐

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature	/Kevin Weddington/	Date Considered	03/03/2010

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11776329	NIYIKIZA ET AL.
	Examiner	Art Unit
	Kevin E Weddington	1614

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	02/11/2009	09/01/2009	01/28/2010	02/23/2010					
	1			-	-					
	2			-	-					
	3			-	-					
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	35			-	-					
	36			-	-					

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11776329	NIYIKIZA ET AL.
	Examiner	**Art Unit**
	Kevin E Weddington	1614

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	02/11/2009	09/01/2009	01/28/2010	02/23/2010					
	37			-	-					
	38			-	-					
	39			-	-					
1	40	✓	✓	✓	=					
2	41	✓	✓	✓	=					
3	42	✓	✓	✓	=					
4	43	✓	✓	✓	=					
5	44	✓	✓	✓	=					
	45	✓	✓	-	-					
	46	✓	✓	-	-					
6	47	✓	✓	✓	=					
7	48	✓	✓	✓	=					
8	49	✓	✓	✓	=					
9	50	✓	✓	✓	=					
10	51	✓	✓	✓	=					
11	52	✓	✓	✓	=					
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13	54			✓	=					
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15	56			✓	=					
16	57			✓	=					
17	58			✓	=					
18	59			✓	=					
19	60			✓	=					
20	61			✓	=					
21	62			✓	=					
22	63			✓	=					

Issue Classification

Application/Control No.	Applicant(s)/Patent Under Reexamination
11776329	NIYIKIZA ET AL.
Examiner	**Art Unit**
KEVIN WEDDINGTON	1614

ORIGINAL		INTERNATIONAL CLASSIFICATION															
CLASS	SUBCLASS	CLAIMED										NON-CLAIMED					
514	52	A	6	1	K	31 / 70 (2006.01.01)											
CROSS REFERENCE(S)		A	6	1	K	31 / 685 (2006.01.01)											
		A	6	1	K	31 / 50 (2006.01.01)											
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)	A	6	1	K	31 / 525 (2006.01.01)											
514	77 249 251 265.1	A	6	1	K	31 / 519 (2006.01.01)											

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
	1		17		33	8	49								
	2		18		34	9	50								
	3		19		35	10	51								
	4		20		36	11	52								
	5		21		37	12	53								
	6		22		38	13	54								
	7		23		39	14	55								
	8		24	1	40	15	56								
	9		25	2	41	16	57								
	10		26	3	42	17	58								
	11		27	4	43	18	59								
	12		28	5	44	19	60								
	13		29		45	20	61								
	14		30		46	21	62								
	15		31	6	47	22	63								
	16		32	7	48										

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	22	
/KEVIN WEDDINGTON/ Primary Examiner.Art Unit 1614	02/23/2010	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

PTO/SB/08a (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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	1					

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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							☐

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11776329
	Filing Date	2007-07-11
	First Named Inventor	Clet Niyikiza
	Art Unit	1614
	Examiner Name	
	Attorney Docket Number	X14173B

/K.W./	1	ALIMTA, NDA 021462, Approved Label of 07/02/2009.	☐
	2	"Clinical Chemistry: principle, procedures, correlations," 3rd edition, 1996, published by Lippincott: pp. 618-627.	☐
	3	Fluorouracil, Physicians Desk References, (c) 1998, pp 2463-2464.	☐
	4	HAMMOND, L., et al., "A phase I and pharmacokinetic (PK) study of the multitarget antifol (MTA) LY231514 with folic acid, " American Society of Clinical Oncology (ASCO) Meeting Abstract No. 866 (1998).	☐
	5	KISLIUK, RL., 1984. "The Biochemistry of Folates." In Sirotnak (Ed.), Folate Antagonists as Therapeutic Agents. pp. 2-68. Harcourt Brace Jovanovich, Publishers.	☐
	6	KISLIUK, RL., 1999. "Folate Biochemistry in RElation to Antifolate Selectivity." In Jackson (Ed.), Antifolate Drugs in Cancer Therapy. pp 13-36. Humana Press, New Jersey.	☐
	7	Leucovorin, Physicians Desk Reference, (c) 1999. pp 1389-1391.	☐
	8	Methotrexate, Physicians Desk Reference, (c) 1999. pp. 1397-1413.	☐
	9	MORGAN, et al., "Folic acid supplementation prevent deficient blood folate levels and hyperhomocysteinemia during long-term, low dose methotrexate therapy for rheumatoid arthritis: implications for cardiovascular disease prevent," J. Rheumatol. 25:441-446. (1998).	☐
	10	NIYIKIZA, C., et al., "LY231514 (MTA): relationship of vitamin metabolite profile to toxicity," American Society of Clinical Oncology (ASCO) Meeting Abstract No. 2139 (1998).	☐
/K.W./	11	Raltitrexed, The Complete Drug Reference, Martindale, 32nd Ed., Pharmaceutical Press, London, pp 560. 1990	☐

/Kevin Weddington/

02/26/2010

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11776329
	Filing Date	2007-07-11
	First Named Inventor	Clet Niyikiza
	Art Unit	1614
	Examiner Name	
	Attorney Docket Number	X14173B

/K.W./	12	SHIH, C., et al., "LY231514, a Pyrrolo[2,3-d]pyrimidine-based Antifolate that Inhibits Multiple Folate-requiring Enzymes," Cancer Research. 57:1116-1123. 1997.	☐
/K.W./	13	SHIH, C., et al., "Preclinical Pharmacology Studies and the Clinical Development of a Novel Multitargeted Antifolate, MTA (LY231514)," In Jackson (Ed.), Antifolate Drugs in Cancer Therapy. pp 13-36. Humana Press, New Jersey. 1998	☐
/K.W./	14	VOLKOV, I., "The master key effect of vitamin B12 in treatment of malignancy - A potential therapy?", Medical Hypotheses. 70:324-328. 2008.	☐
	15		☐

If you wish to add additional non-patent literature document citation information please click the Add button [Add]

EXAMINER SIGNATURE

Examiner Signature	/Kevin Weddington/	Date Considered	02/26/2010

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

Bib Data Sheet

CONFIRMATION NO. 6568

SERIAL NUMBER	FILING OR 371(c) DATE / RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/776,329	07/11/2007	514	1614	X14173B

APPLICANTS

Clet Niyikiza, Indianapolis, IN;

**** CONTINUING DATA ***************************

This application is a DIV of 11/288,807 11/29/2005 ABN
which is a DIV of 10/297,821 12/05/2002 PAT 7,053,065
which is a 371 of PCT/US01/14860 06/15/2001
which claims benefit of 60/215,310 06/30/2000
and claims benefit of 60/235,859 09/27/2000 ABN
and claims benefit of 60/284,448 04/18/2001

**** FOREIGN APPLICATIONS *********************

IF REQUIRED, FOREIGN FILING LICENSE GRANTED
** 08/31/2007

Foreign Priority claimed	STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ yes ☐ no	IN	0	11	2

35 USC 119 (a-d) conditions met ☐ yes ☐ no ☐ Met after Allowance

Verified and Acknowledged _____ Examiner's Signature Initials

ADDRESS
25885

TITLE

NOVEL ANTIFOLATE COMBINATION THERAPIES

FILING FEE RECEIVED		
1546	FEES: Authority has been given in Paper No. _____ to charge/credit DEPOSIT ACCOUNT No. _____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

cwc
4/16/10

PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Applicant:	NIYIKIZA Clet	Group Art Unit: 1614
Serial No.:	11/776329	Examiner: Weddington, Kevin E.
Application Date:	July 11, 2007	Confirmation No.: 6568
For:	NOVEL ANTIFOLATE COMBINATION THERAPIES	
Docket No.:	X14173B	

COMMUNICATION - REMINDER AT TIME OF ISSUE OF CHANGE OF INVENTORSHIP

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450
Attention: Mail Stop Issue Fee

Sir:

The above-captioned application has been allowed. In the Notice of Allowance and Issue Fee Due, the first named Applicant is identified as Clet Niyikiza. Clet Niyikiza is the first of three named Applicants: Clet Niyikiza, Paolo Paoletti, and James Jacob Rusthoven in the original filing of this application. However, a Petition to Correct Inventorship was submitted July 11, 2007, removing Applicants Paolo Paoletti and James Jacob Rusthoven.

Accordingly, we ask that the proper steps be taken to ensure that the patent issues solely in the name of Clet Niyikiza.

Respectfully submitted,
/Elizabeth A McGraw/
Elizabeth A. McGraw
Attorney for Applicants
Registration No. 44,646
Phone: 317-277-7443

Eli Lilly and Company
Patent Division
P.O. Box 6288
Indianapolis, Indiana 46206-6288
April 26, 2010

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

25885 7590 03/10/2010

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/776,329	07/11/2007	Clet Niyikiza	X14173B	6568

TITLE OF INVENTION: NOVEL ANTIFOLATE COMBINATION THERAPIES

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1510	$300	$0	$1810	06/10/2010

EXAMINER	ART UNIT	CLASS-SUBCLASS
WEDDINGTON, KEVIN E	1614	514-052000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Elizabeth A. McGraw
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Eli Lilly and Company

(B) RESIDENCE: (CITY and STATE OR COUNTRY): Indianapolis, Indiana

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
- ☒ Issue Fee
- ☒ Publication Fee (No small entity discount permitted)
- ☐ Advance Order - # of Copies .....

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
- ☐ A check is enclosed.
- ☐ Payment by credit card. Form PTO-2038 is attached.
- ☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number 05-0840 (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
- ☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.
- ☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature [signature] Date 22 Apr 2010

Typed or printed name Elizabeth McGraw Registration No. 44,646

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Electronic Patent Application Fee Transmittal

Application Number:	11776329
Filing Date:	11-Jul-2007
Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Filer:	Elizabeth Ann McGraw/Linda Durbin
Attorney Docket Number:	X14173B

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Utility Appl issue fee	1501	1	1510	1510
Publ. Fee- early, voluntary, or normal	1504	1	300	300

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1810**

Electronic Acknowledgement Receipt

EFS ID:	7485297
Application Number:	11776329
International Application Number:	
Confirmation Number:	6568
Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Customer Number:	25885
Filer:	Elizabeth Ann McGraw/Linda Durbin
Filer Authorized By:	Elizabeth Ann McGraw
Attorney Docket Number:	X14173B
Receipt Date:	26-APR-2010
Filing Date:	11-JUL-2007
Time Stamp:	13:47:13
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1810
RAM confirmation Number	9928
Deposit Account	050840
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Issue Fee Payment (PTO-85B)	X14173BIssueFeeTransmittal.pdf	375077 c0268b10a75768a1ebed7efd7501c3e70d891525	no	1
Warnings:					
Information:					
2	Post Allowance Communication - Incoming	X14173BInventorshipReminder.pdf	63107 776e9a2738837599a42d628ebd80f93388fdc8be	no	1
Warnings:					
Information:					
3	Fee Worksheet (PTO-875)	fee-info.pdf	32306 e4cfbb479aeedbf5315951f2ca4bb092624004ed	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	470490	

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<u>New Applications Under 35 U.S.C. 111</u>
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

OK TO ENTER: /K.W./
05/24/2010

CERTIFICATION OF FACSIMILE TRANSMISSION
I hereby certify that this paper is being facsimile transmitted to the Patent and Trademark Office on the date shown below.
Type or print name of person signing certification
Signature Date

PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Applicant:	NIYIKIZA Clet	
For:	NOVEL ANTIFOLATE COMBINATION THERAPIES	
Docket No.:	X-14173B	

AMENDMENT AND PETITION TO CORRECT INVENTORSHIP UNDER 37 C.F.R. 1.48(b)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. Amendment and Petition

This amendment and petition is to delete the names of the following persons originally named as inventors and who are not the inventors of the invention now being claimed: Paolo Paoletti, of Indianapolis, Indiana, and James Jacob Rusthoven, of Ancaster, Canada.

2. Claims Now On File

The claims in this application are as follows:

New claims 29-39 filed on July 11, 2007

3. Diligence

This amendment and petition is being filed diligently after discovery that any claims for which the above named inventors who are being deleted are now no longer the inventors of the subject matter being claimed.

4. Fee Payment

Please charge $130.00, the surcharge required by §1.17(i), and charge any additional fees which may be required by this or any other related paper, or credit any overpayment to Deposit Account No. 05-0840, in the name of Eli Lilly and Company. I enclose an original and two copies of this paper.

Respectfully submitted,

/Manisha A. Desai/
Manisha A. Desai, Ph.D.
Attorney for Applicant
Registration No. 43,585
Telephone: (317) 433-5333

Eli Lilly and Company
Patent Division
P.O. Box 6288
Indianapolis, Indiana 46206-6288

July 11, 2007

FORM PTO 1449 (modified) INFORMATION DISCLOSURE CITATION IN AN APPLICATION	Atty. Docket No. X-14173B	Serial No 11/776,329
	First Applicant NIYIKIZA Clet	
	Filing Date	Group

6/7/10 ES

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Pages or Relevant Figures Appear
/KW/	AA	US 5,405,839	4/11/1995	~~Tetsuo~~, et al. Toraya	
	AB	US 5,431,925	07/00/1995	Ohmori, et al.	
	AC	US 5,563,126	10/8/1996	Allen, et al.	
	AD	US 5,736,402	4/7/1998	Francis, et al.	
	AE	US 6,207,651	3/27/2001	Allen, et al.	
	AF	US 6,297,224	10/2/2001	Allen, et al.	
	AG	US 6,528,496	3/4/2003	Allen, et al.	
	AH	US 03/0216350	11/20/2003	Allen, et al.	
	AI	US 03/0225030	12/4/2003	Allen, et al.	
	AJ	US 2,920,015	01/1960	Thompson, Robert E.	
	AK	US 2004/0005311 A1	01/2004	Pitman, Bradford D.	
	AL	US 5,344,932	09/1994	Taylor, Edward C.	
/KW/	AM	US 7,053,065	05/2006	Niyikiza, et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code5 (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Line., Where Relevant Passages or Relevant Figures Appe:	T[6]
/KW/	BA	EP 0 546 870	6/16/1993	EPO		

Examiner Signature	/Kevin Weddington/ (02/11/2009)	Date Considered	02/11/2009

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3]Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4]For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5]Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST. 16 if possible. [6]Applicant is to place a check mark here if English language Translation is attached.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/776,329	07/11/2007	Clet Niyikiza	X14173B	6568

25885 7590 07/13/2010

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

EXAMINER
WEDDINGTON, KEVIN E

ART UNIT	PAPER NUMBER
1614	

NOTIFICATION DATE	DELIVERY MODE
07/13/2010	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patents@lilly.com

UNITED STATES DEPARTMENT OF COMMERCE
U.S. Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450

APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
11776329	7/11/2007	NIYIKIZA, CLET	X14173B

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

EXAMINER
KEVIN WEDDINGTON

ART UNIT	PAPER
1614	20100706

DATE MAILED:

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner for Patents

In view of the papers filed July 11, 2007, the inventorship in this nonprovisional application has been changed by the deletion of Paolo Paoletti and James Jacob Rusthoven.

The solely applicant is Clet Niyikiza.

/KEVIN WEDDINGTON/
Primary Examiner
Art Unit: 1614

PTO-90C (Rev.04-03)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
11/776,329	07/11/2007	1614	1846	X14173B	11	2

CONFIRMATION NO. 6568

CORRECTED FILING RECEIPT

25885
ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

OC000000042552942

Date Mailed: 07/14/2010

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Clet Niyikiza, Indianapolis, IN;

Power of Attorney: The patent practitioners associated with Customer Number 25885

Domestic Priority data as claimed by applicant

This application is a DIV of 11/288,807 11/29/2005 ABN
which is a DIV of 10/297,821 12/05/2002 PAT 7,053,065
which is a 371 of PCT/US01/14860 06/15/2001
which claims benefit of 60/215,310 06/30/2000
and claims benefit of 60/235,859 09/27/2000 ABN
and claims benefit of 60/284,448 04/18/2001

Foreign Applications

If Required, Foreign Filing License Granted: 08/31/2007

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 11/776,329**

Projected Publication Date: Not Applicable

Non-Publication Request: No

Early Publication Request: No

Title

NOVEL ANTIFOLATE COMBINATION THERAPIES

Preliminary Class

514

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as

set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

Bib Data Sheet

CONFIRMATION NO. 6568

SERIAL NUMBER	FILING OR 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/776,329	07/11/2007 RULE	514	1614	X14173B

APPLICANTS

Clet Niyikiza, Indianapolis, IN;

**** CONTINUING DATA ***************************

This application is a DIV of 11/288,807 11/29/2005 ABN
which is a DIV of 10/297,821 12/05/2002 PAT 7,053,065
which is a 371 of PCT/US01/14860 06/15/2001
which claims benefit of 60/215,310 06/30/2000
and claims benefit of 60/235,859 09/27/2000 ABN
and claims benefit of 60/284,448 04/18/2001

**** FOREIGN APPLICATIONS *********************

IF REQUIRED, FOREIGN FILING LICENSE GRANTED
** 08/31/2007

Foreign Priority claimed	STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ yes ☐ no	IN	0	11	2
35 USC 119 (a-d) conditions met ☐ yes ☐ no ☐ Met after Allowance				
Verified and Acknowledged Examiner's Signature Initials				

ADDRESS
25885

TITLE

NOVEL ANTIFOLATE COMBINATION THERAPIES

FILING FEE RECEIVED		
1846	FEES: Authority has been given in Paper No. _____ to charge/credit DEPOSIT ACCOUNT No. _____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/776,329	08/10/2010	7772209	X14173B	6568

25885 7590 07/21/2010

ELI LILLY & COMPANY
PATENT DIVISION
P.O. BOX 6288
INDIANAPOLIS, IN 46206-6288

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 162 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Clet Niyikiza, Indianapolis, IN;

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

U. S. Patent No.	:	7,772,209
Issued:	:	August 10, 2010
First Applicant	:	Clet Niyikiza
Serial No.	:	11/776,329
Application Date	:	July 11, 2007
Entitled	:	Antifolate Combination Therapies
Docket No.	:	X14173B

REQUEST FOR CERTIFICATE OF CORRECTION UNDER 37 C.F.R. 1.322

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The patentee of the above-identified patent respectfully requests that you issue a Certificate of Correction to correct errors in the printed patent. Attached is Form PTO 1050 on which the errors are specified.

Some of the errors are typographical and were made inadvertently. The remaining errors occurred during the printing of the patent.

Please charge the fee under 1.20(a) and charge any additional fees which may be required by this or any other related paper, or credit any overpayment to Deposit Account No. 05-0840 to cover the cost of this Certificate of Correction.

Respectfully submitted,

/Elizabeth A. McGraw/
Elizabeth A. McGraw
Attorney for Applicant
Registration No. 44,646
Phone: 317-277-7443

Eli Lilly and Company
Patent Division
P.O. Box 6288
Indianapolis, Indiana 46206-6288

September 20, 2010

PTO/SB/44 (09-07)
Approved for use through 08/31/2010. OMB 0651-0033
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 7,772,209

APPLICATION NO.: 11/776,329

ISSUE DATE : August 10, 2010

INVENTOR(S) : Clet Niyikiza

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

First Page, Col. 2, Line 22, under Other Publications: Delete "Homocystein" and insert --Homocysteine--, therefor.

First Page, Col. 2, Line 27, under Other Publications: Delete "hydroxocobaltniin" and insert --hydroxocobalamin--, therefor.

First Page, Col. 2, Line 28, under Other Publications: Delete "mce" and insert --mice--, therefor.

First Page, Col. 2, Line 37, under Other Publications: Delete "2666" and insert --266--, therefor.

Column 1, Line 5: Delete "12 May," and insert --5 Dec.--, therefor.

Column 10, Line 62: In Claim 1, delete "hydroxycobalamin," and insert --hydroxocobalamin,--, therefor.

Column 11, Line 4: In Claim 4, delete "2," and insert --3,--, therefor.

MAILING ADDRESS OF SENDER (Please do not use customer number below):

Eli Lilly and Company
P.O. Box 6288
Indianapolis, IN 46206-6288

Electronic Acknowledgement Receipt

EFS ID:	8464324
Application Number:	11776329
International Application Number:	
Confirmation Number:	6568
Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Customer Number:	25885
Filer:	Elizabeth Ann McGraw/Linda Durbin
Filer Authorized By:	Elizabeth Ann McGraw
Attorney Docket Number:	X14173B
Receipt Date:	21-SEP-2010
Filing Date:	11-JUL-2007
Time Stamp:	15:28:58
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$100
RAM confirmation Number	1875
Deposit Account	050840
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Certificate of Correction	X14173BRequestCertificateofCorrection.pdf	276775 3dfd3cab0967543cd0618f3e2c32e60ff5671bd0	no	2
Warnings:					
Information:					
2	Fee Worksheet (PTO-875)	fee-info.pdf	30372 23f9dc93ad89b23edb112ce21d94211041f77577	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	307147		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

<u>New Applications Under 35 U.S.C. 111</u>
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Patent Application Fee Transmittal

Application Number:	11776329
Filing Date:	11-Jul-2007
Title of Invention:	NOVEL ANTIFOLATE COMBINATION THERAPIES
First Named Inventor/Applicant Name:	Clet Niyikiza
Filer:	Elizabeth Ann McGraw/Linda Durbin
Attorney Docket Number:	X14173B

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Certificate of correction	1811	1	100	100
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**100**

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,772,209 B2
APPLICATION NO. : 11/776329
DATED : August 10, 2010
INVENTOR(S) : Clet Niyikiza

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title Page, Col. 2, Line 22, under Other Publications: Delete "Homocystein" and insert --Homocysteine--, therefor.

Title Page, Col. 2, Line 27, under other Publications: Delete "hydroxocobaltniin" and insert --hydroxocobalamin--, therefor.

Title Page, Col. 2, Line 28, under Other Publications: Delete "mce" and insert --mice--, therefor.

Title Page, Col. 2, Line 37, under Other Publications: Delete "2666" and insert --266--, therefor.

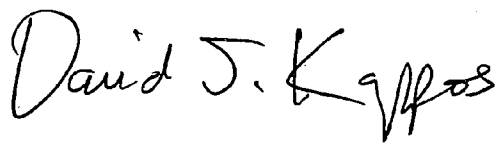
Column l, Line 5, Delete "12 May," and insert --5 Dec.--, therefor.

Column 10, Line 62, In Claim l, delete "hydroxycobalamin," and insert --hydroxocobalamin,--, therefor.

Column 11, Line 4, In Claim 4, delete "2," and insert --3,--, therefor.

Signed and Sealed this

Twenty-sixth Day of October, 2010



David J. Kappos
Director of the United States Patent and Trademark Office

AO 120 (Rev. 3/04)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court Southern District of Indiana on the following G Patents or G Trademarks:

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:10-cv-1376-TWP-DML	10/29/2010	Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	TEVA PARENTERAL MEDICINES, INC., APP PHARMACEUTICALS, LLC, PLIVA HRVATSKA D.O.O., TEVA PHARMACEUTICALS USA INC., and BARR LAB

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772,209 B2	8/10/2010	CLET NIYIKIZA, Inventor
2			
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5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY G Amendment G Answer G Cross Bill G Other Pleading	
	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772, 209 B2	8/10/2010	***SEE ATTACHED COMPLAINT FILED ON 10/29/2010***
2			
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
		11/2/2010

Copy 1—Upon initiation of action, mail this copy to Director Copy 3—Upon termination of action, mail this copy to Director
Copy 2—Upon filing document adding patent(s), mail this copy to Director Copy 4—Case file copy

AO 120 (Rev. 3/04)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court Southern District of Indiana on the following ☑ Patents or G Trademarks:

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:10-cv-1376-TWP-DML	10/29/2010	Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	TEVA PARENTERAL MEDICINES, INC., APP PHARMACEUTICALS, LLC, PLIVA HRVATSKA D.O.O., TEVA PHARMACEUTICALS USA INC., and BARR LAB

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772,209 B2	8/10/2010	CLET NIYIKIZA, Inventor
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In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY G Amendment ☑ Answer G Cross Bill Other Pleading	
	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1			***SEE ATTACHED ANSWER FILED ON 2/7/11***
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
Laura A. Briggs	Eldon Dowson	2/14/2011

Copy 1—Upon initiation of action, mail this copy to Director Copy 3—Upon termination of action, mail this copy to Director
Copy 2—Upon filing document adding patent(s), mail this copy to Director Copy 4—Case file copy

AO 120 (Rev. 3/04)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court Southern District of Indiana on the following ☑ Patents or ☐ Trademarks:

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:10-cv-1376-TWP-DML	10/29/2010	Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	TEVA PARENTERAL MEDICINES, INC., APP PHARMACEUTICALS, LLC, PLIVA HRVATSKA D.O.O., TEVA PHARMACEUTICALS USA INC., and BARR LAB

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772,209 B2	8/10/2010	CLET NIYIKIZA, Inventor
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In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☑ Answer ☐ Cross Bill Other Pleading	
	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1			**SEE ATTACHED ANSWER FILED ON 2/22/2011**
2			
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
Laura A. Briggs	Adam Dobson	2/28/2011

Copy 1—Upon initiation of action, mail this copy to Director Copy 3—Upon termination of action, mail this copy to Director
Copy 2—Upon filing document adding patent(s), mail this copy to Director Copy 4—Case file copy

AO 120 (Rev. 3/04)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court Southern District of Indiana on the following G Patents or G Trademarks:

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:11-cv-942-TWP-TAB	7/15/2011	Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	APP PHARMACEUTICALS, LLC

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772,209	8/10/2010	**SEE ATTACHED COMPLAINT**
2			
3			
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5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY G Amendment G Answer G Cross Bill G Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
Laura A. Briggs	Jordan Dobson	7/25/2011

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 3/04)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court Southern District of Indiana on the following ☑ Patents or G Trademarks:

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:10-cv-1376-TWP-DML	10/29/2010	Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	TEVA PARENTERAL MEDICINES, INC., APP PHARMACEUTICALS, LLC, PLIVA HRVATSKA D.O.O., TEVA PHARMACEUTICALS USA INC., and BARR LAB

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772,209 B2	8/10/2010	CLET NIYIKIZA, Inventor
2			
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In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY G Amendment ☑ Answer G Cross Bill Other Pleading	
	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1			
2			**See attached Answer to Complaint filed in
3			Consolidated Case 1:11-cv-942-TWP-TAB.**
4			
5			

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
Laura A. Briggs	Adam Dobson	9/26/2011

Copy 1—Upon initiation of action, mail this copy to Director Copy 3—Upon termination of action, mail this copy to Director
Copy 2—Upon filing document adding patent(s), mail this copy to Director Copy 4—Case file copy

AO 120 (Rev. 3/04)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court Southern District of Indiana on the following G Patents or G Trademarks:

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:11-cv-942-TWP-TAB	7/15/2011	Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	APP PHARMACEUTICALS, LLC

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772,209	8/10/2010	
2			
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In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY G Amendment G Answer G Cross Bill G Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT
See attached Order of Consolidation.

CLERK	(BY) DEPUTY CLERK	DATE
Laura A. Briggs	Adam Dobson	9/12/2011

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 3/04)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court Southern District of Indiana on the following ☑ Patents or G Trademarks:

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:10-cv-1376-P/L	10/29/2010	Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	TEVA PARENTERAL MEDICINES, INC., APP PHARMACEUTICALS, LLC, PLIVA HRVATSKA D.O.O., TEVA PHARMACEUTICALS USA INC., and BARR LAB

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772,209	8/10/2010	
2			
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In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY
9/25/2012	☑ Amendment G Answer G Cross Bill G Other Pleading

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1			**SEE ATTACHED AMENDED COMPLAINT**
2			
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
Tamara Biggs	Jordan Dawson	10/2/2012

Copy 1—Upon initiation of action, mail this copy to Director **Copy 3—Upon termination of action, mail this copy to Director**
Copy 2—Upon filing document adding patent(s), mail this copy to Director **Copy 4—Case file copy**

AO 120 (Rev. 3/04)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court Southern District of Indiana on the following ☑ Patents or G Trademarks:

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:13-cv-00335-TWP-DK	2/28/2013	Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	ACCORD HEALTHCARE INC., USA

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772,209		
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In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY G Amendment G Answer G Cross Bill G Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
Laura A. Briggs	Adam Dawson	3/11/2013

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DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:13-cv-335-TWP-DKL	2/28/2013	Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	ACCORD HEALTHCARE INC., USA

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772,209		
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In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY
6/24/2013	G Amendment G Answer G Cross Bill G Other Pleading

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT
ORDER OF CONSOLIDATION - This cause of action is hereby consolidated under action 1:12-cv-86-TWP-DKL.

CLERK	(BY) DEPUTY CLERK	DATE
Laura A. Briggs		7/1/2013

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Trials@uspto.gov
571-272-7822

Paper No. 13
Date Entered: October 1, 2013

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ACCORD HEALTHCARE, INC., USA
Petitioner

v.

ELI LILLY & COMPANY
Patent Owner

Case IPR2013-00356
Patent 7,772,209

Before MICHAEL J. FITZPATRICK, RAMA G. ELLURU, and SCOTT E. KAMHOLZ, *Administrative Patent Judges.*

KAMHOLZ, *Administrative Patent Judge.*

DECISION
Denying *Inter Partes* Review
37 C.F.R. § 42.108

I. INTRODUCTION

Accord Healthcare, Inc., USA ("Accord") filed a petition (Paper 4) on June 14, 2013 to institute an *inter partes* review of claims 1-22 of U.S. Patent 7,772,209 ("the '209 patent"). Accord later filed a corrected petition (Paper 6, "Pet."). Patent Owner Eli Lilly & Company ("Eli Lilly") filed a preliminary response (Paper 10, "Prelim. Resp."). The Board, acting on behalf of the Director, has jurisdiction under 35 U.S.C. § 314.

The '209 patent is involved in several civil actions for patent infringement, including *Eli Lilly & Co. v. Accord Healthcare, Inc., USA et al.*, 1:12-cv-00086-TWP-DKL (S.D. Ind.) ("the '086 action"), filed January 20, 2012 and served January 23, 2012, and *Eli Lilly & Co. v. Accord Healthcare, Inc., USA,* 1:13-cv-00335-TWP-DKL (S.D. Ind.) ("the '335 action"), filed February 28, 2013 and served March 7, 2013. Pet. 1; Prelim. Resp. 5-6.* The '335 action has been consolidated into the '086 action. Prelim. Resp. 6-7.

We deny the petition because it is time-barred under 35 U.S.C. § 315(b).

II. ANALYSIS

Eli Lilly served Accord with a complaint alleging infringement of the '209 patent on at least two occasions: the '086 action, on January 23, 2012, and the '355 action, on March 7, 2013. Ex. 2004 (return of service for the '086 action); Prelim. Resp. 5-6; *see also* Pet. 1. The earlier complaint was served more than one year before Accord filed the present petition; the latter, less than one year.

* The parties disagree as to whether the complaint in the '355 action was served on February 28, 2013 or March 7, 2013. For purposes of this decision, we accept Eli Lilly's representation that the complaint was served on March 7, 2013.

Section 315(b) of Title 35 of the United States Code provides:

> (b) PATENT OWNER'S ACTION.—An inter partes review may not be instituted if the petition requesting the proceeding is filed more than 1 year after the date on which the petitioner, real party in interest, or privy of the petitioner is served with a complaint alleging infringement of the patent. The time limitation set forth in the preceding sentence shall not apply to a request for joinder under subsection (c).

Accord argues that its petition is timely because it was filed less than one year after the date on which it was served with a complaint in the '355 action. Pet. 2-3. Accord acknowledges service on January 23, 2012 of a complaint in the '086 action, but argues that the two infringement actions concern distinct products and are based on different sets of facts. *Id.* at 3 n.1.

We reject Accord's implicit argument that the one-year period set forth in § 315(b) should not be measured from the date of service of the complaint in the '086 action. The plain language of the statute does not indicate or suggest that the filing of a later lawsuit renders the service of a complaint in an earlier lawsuit a nullity. Moreover, as the legislative history of 35 U.S.C. § 315(b) indicates, Congress intended that *inter partes* reviews should not be used as "tools for harassment" by "repeated litigation and administrative attacks." H.R.Rep. No. 112-98 at 48 (2011). Allowing such attacks "would frustrate the purpose of the section as providing quick and cost effective alternatives to litigation." *Id.*

Accord was "served with a complaint alleging infringement of the patent" on January 23, 2012. Ex. 2004. The petition was filed more than one year after that date and is, therefore, barred. *See Universal Remote Control, Inc. v. Universal Elec., Inc.*, IPR2013-00168, Paper 9 at 4 (PTAB Aug. 26, 2013).

III. CONCLUSION

The Board denies the petition because it was not filed within the time limit imposed by 35 U.S.C. § 315(b).

IV. ORDER

For the reasons given, it is

ORDERED that the petition challenging the patentability of claims 1-22 of U.S. Patent 7,772,209 is *denied.*

IPR2013-00356
Patent 7,772,209

For Petitioner:

Chidambaram S. Iyer
Chandran B. Iyer
Sughrue Mion PLLC

For Patent Owner:

Andrew V. Trask
Williams & Connolly LLP

Mark J. Stewart
Eli Lilly & Company

AO 120 (Rev. 3/04)

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DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:13-cv-1469-TWP-DML	9/13/2013	Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	SUN PHARMACEUTICAL INDUSTRIES LTD.; SUN PHARMA GLOBAL FZE

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772,209	8/10/2010	ELI LILLY AND COMPANY
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In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY G Amendment G Answer G Cross Bill G Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
Tamara Riggs	Adam Dawson	9/17/2013

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AO 120 (Rev. 08/10)

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☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:14-104-TWP-DKL	1/23/2014	for the Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	GLENMARK GENERICS INC., USA GLENMARK PHARMACEUTICALS LTD GLENMARK GENERICS LTD.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,7772.209	8/10/2010	ELI LILLY AND COMPANY
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In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
Laura A. Briggs		1/23/2014

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DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1:10-cv-1376-TWP-DML	10/29/2010	Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	TEVA PARENTERAL MEDICINES, INC., APP PHARMACEUTICALS, LLC, PLIVA HRVATSKA D.O.O., TEVA PHARMACEUTICALS USA INC., and BARR LAB

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
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In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☑ Other Pleading		
	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772, 209 B2	8/10/2010	CLET NIYIKIZA, Inventor
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT
Closed Judgment dated 3/31/2014, see attached.

CLERK	(BY) DEPUTY CLERK	DATE
Lawrence A. Briggs	Martha L. Gutierrez	4/29/2014

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G Trademarks or ☑ Patents. (G the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
1"15-cv-1083-B-K	7/10/2015	Southern District of Indiana

PLAINTIFF	DEFENDANT
ELI LILLY AND COMPANY	MYLAN LABORATORIES LIMITED, MYLAN INC., and MYLAN PHARMACEUTICALS INC.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	7,772,209		**see copy of Complaint attached**
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In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY G Amendment G Answer G Cross Bill G Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
Laura A. Briggs	Melanie Carmichael	7/20/2015

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In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court Eastern District of Wisconsin on the following

☑ Trademarks or ☐ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
15-C-869	7/17/2015	Eastern District of Wisconsin

PLAINTIFF	DEFENDANT
Klement Sausage Co Inc	Johnsonville Sausage LLC

PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
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In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1 3684763	9/22/2009	Klement Sausage Co Inc
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In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE
Jon W. Sanfilippo	s/ Amanda S. Chasteen	7/20/2015

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