PETITIONER'S DEMONSTRATIVES

March 16, 2017
Oral Argument

Neptune Generics, LLC,
Apotex Inc., Apotex Corp., Teva Pharmaceuticals,
Fresenius Kabi USA, LLC, and
Wockhardt Bio AG,

Petitioners,

v.

Eli Lilly & Company,
Patent Owner.

IPR2016-00237[1] -00240[2]

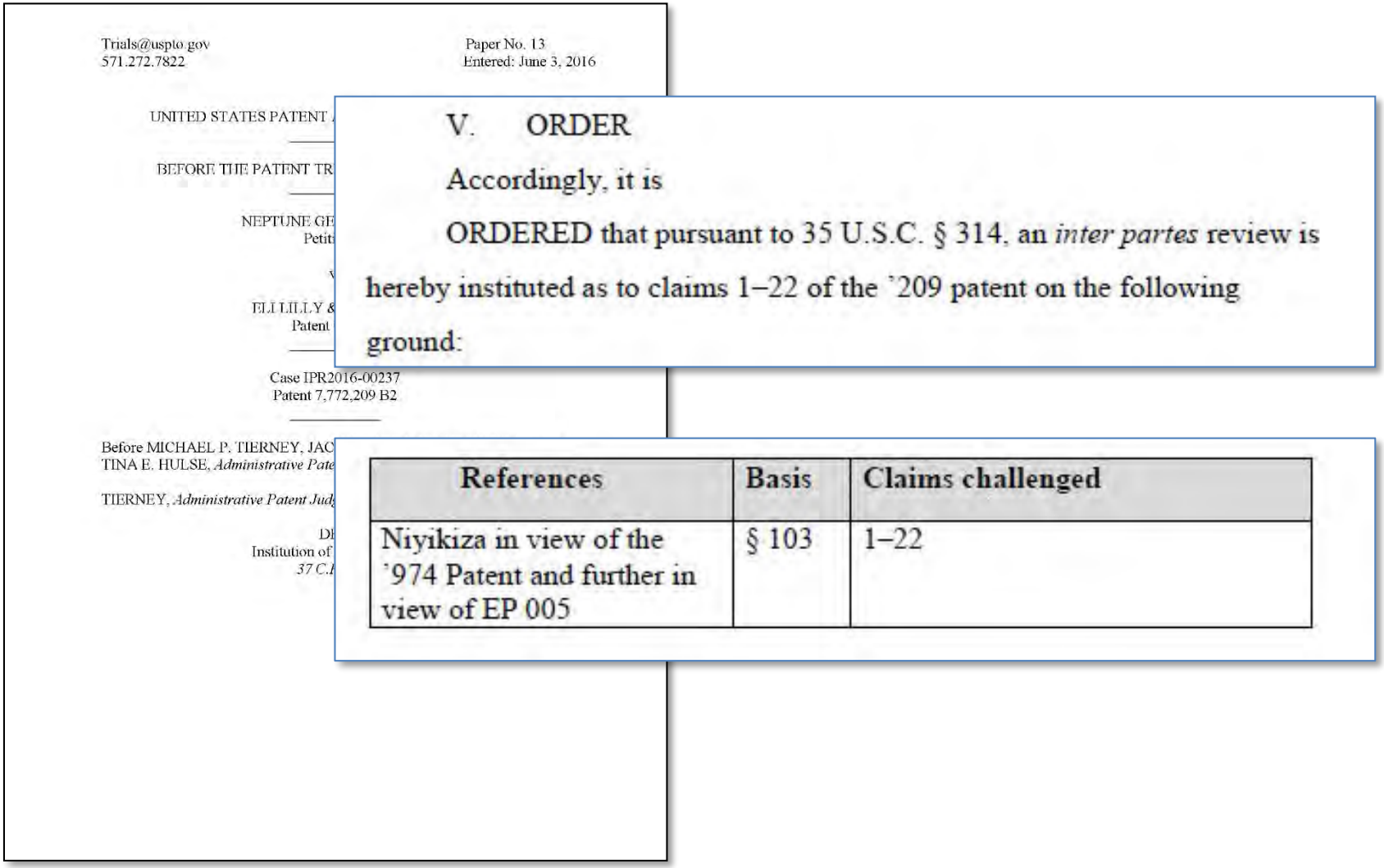
Unless otherwise indicated, all exhibit and paper numbers refer to the -00237 proceeding.

[1]Cases IPR2016-01190, IPR2016-01335 and IPR2016-01341 have been joined with the instant proceeding.
[2]Cases IPR2016-01191, IPR2016-01337 and IPR2016-01343 have been joined with the instant proceeding.

U.S. PATENT No. 7,772,209
INSTITUTION OF IPRS

Grounds for Institution of IPR

Institution Decision -00237



Trials@uspto.gov
571.272.7822

Paper No. 13
Entered: June 3, 2016

UNITED STATES PATENT

BEFORE THE PATENT TR

NEPTUNE GE
Petit

ELI LILLY &
Patent

Case IPR2016-00237
Patent 7,772,209 B2

Before MICHAEL P. TIERNEY, JAC
TINA E. HULSE, *Administrative Pate*

TIERNEY, *Administrative Patent Judg*

DE
Institution of
37 C.

V. ORDER

Accordingly, it is

ORDERED that pursuant to 35 U.S.C. § 314, an *inter partes* review is hereby instituted as to claims 1–22 of the '209 patent on the following ground:

References	Basis	Claims challenged
Niyikiza in view of the '974 Patent and further in view of EP 005	§ 103	1–22

Source: Paper 13 (-00237), Institution Decision, at 19.

Grounds for Institution of IPR

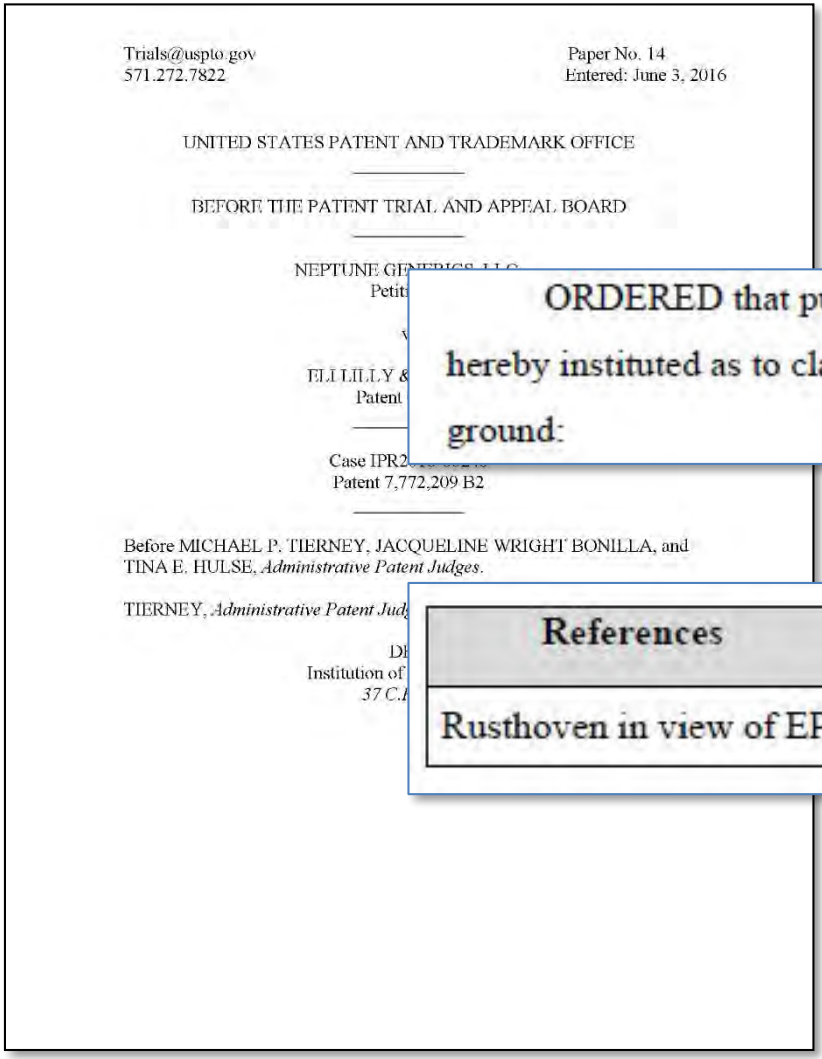
Institution Decision -00237

A limitation on the use of antifolate drugs is "that the cytotoxic activity and subsequent effectiveness of the antifolates may be associated with substantial toxicity for some patients." Ex. 1001, 1:62–64. Homocysteine levels have been shown to be a predictor of cytotoxic events related to the use of certain antifolate enzyme inhibitors. *Id.* at 2:16–26. The '209 patent states that folic acid has been shown to lower homocysteine levels. *Id.* Additionally, the patent states that it was known in the art to treat and prevent cardiovascular disease with a combination of folic acid and vitamin B12. *Id.* at 2:50–54.

Source: Paper 13 (-00237), Institution Decision, at 3-4.

Grounds for Institution of IPR

Institution Decision -00240



Trials@uspto.gov
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Paper No. 14
Entered: June 3, 2016

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

NEPTUNE GE
Petit

ELI LILLY &
Patent

Case IPR2
Patent 7,772,209 B2

Before MICHAEL P. TIERNEY, JACQUELINE WRIGHT BONILLA, and TINA E. HULSE, *Administrative Patent Judges*.

TIERNEY, *Administrative Patent Judg*

DE
Institution of
37 C.

ORDERED that pursuant to 35 U.S.C. § 314, an *inter partes* review is hereby instituted as to claims 1–22 of the '209 patent on the following ground:

References	Basis	Claims challenged
Rusthoven in view of EP 005	§ 103	1–22

Source: Paper 14 (-00240), Institution Decision, at 19.

’209 PATENT

’209 Patent Claims

’209 Patent

1. A method for administering pemetrexed disodium to a patient in need thereof comprising administering an effective amount of folic acid and an effective amount of a methylmalonic acid lowering agent followed by administering an effective amount of pemetrexed disodium, wherein

the methylmalonic acid lowering agent is selected from the group consisting of vitamin B12, hydroxycobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-cobalamin perchlorate, azidocobalamin, cobalamin, cyanocobalamin, or chlorocobalamin.

2. The method of claim **1**, wherein the methylmalonic acid lowering agent is vitamin B12.

3. The method of claim **2**, wherein the vitamin B12 is administered as an intramuscular injection of about 500 μg to about 1500 μg.

4. The method of claim **2**, wherein the vitamin B12 is administered as an intramuscular injection of about 1000 μg.

5. The method of claim **2**, **3** or **4**, wherein the vitamin B12 administration is repeated about every 6 to about every 12 weeks following the administration of vitamin B12 until the administration of the pemetrexed disodium is discontinued.

6. The method of claim **5** wherein the folic acid is administered 1 to 3 weeks prior to the first administration of the pemetrexed disodium.

7. The method of claim **5** wherein the folic acid is administered from about 1 to about 24 hours prior to administration of the pemetrexed disodium.

8. The method according to any one of claims **1-4**, wherein between 0.3 mg to about 5 mg of folic acid is administered orally.

9. The method of claim **8** wherein about 350 μg to about 1000 μg of folic acid is administered.

10. The method of claim **9** wherein 350 μg to 600 μg of folic acid is administered.

11. The method of claim **1** further comprising the administration of cisplatin to the patient.

12. An improved method for administering pemetrexed disodium to a patient in need of chemotherapeutic treatment, wherein the improvement comprises:

a) administration of between about 350 μg and about 1000 μg of folic acid prior to the first administration of pemetrexed disodium;

Source: Ex. 1001 at claims; Paper 1 at *passim*.

’209 Patent Claims

’209 Patent

b) administration of about 500 μg to about 1500 μg of vitamin B12, prior to the first administration of pemetrexed disodium; and

c) administration of pemetrexed disodium.

13. The method of claim **12** further comprising the administration of cisplatin to the patient.

14. The method of claim **12**, wherein vitamin B12 is administered as an intramuscular injection of about 500 μg to about 1500 μg.

15. The method of claim **14**, wherein vitamin B12 is administered as an intramuscular injection of about 1000 μg.

16. The method of claim **15**, wherein between 0.3 mg to about 5 mg of folic acid is administered orally.

17. The method of claim **16** wherein about 350 μg to about 1000 μg of folic acid is administered.

18. The method of claim **17** wherein 350 μg to 600 μg of folic acid is administered.

19. The method of claim **18** wherein folic acid is administered 1 to 3 weeks prior to the first administration of the pemetrexed disodium.

20. The method of claim **18** wherein the folic acid is administered from about 1 to about 24 hours prior to administration of the pemetrexed disodium.

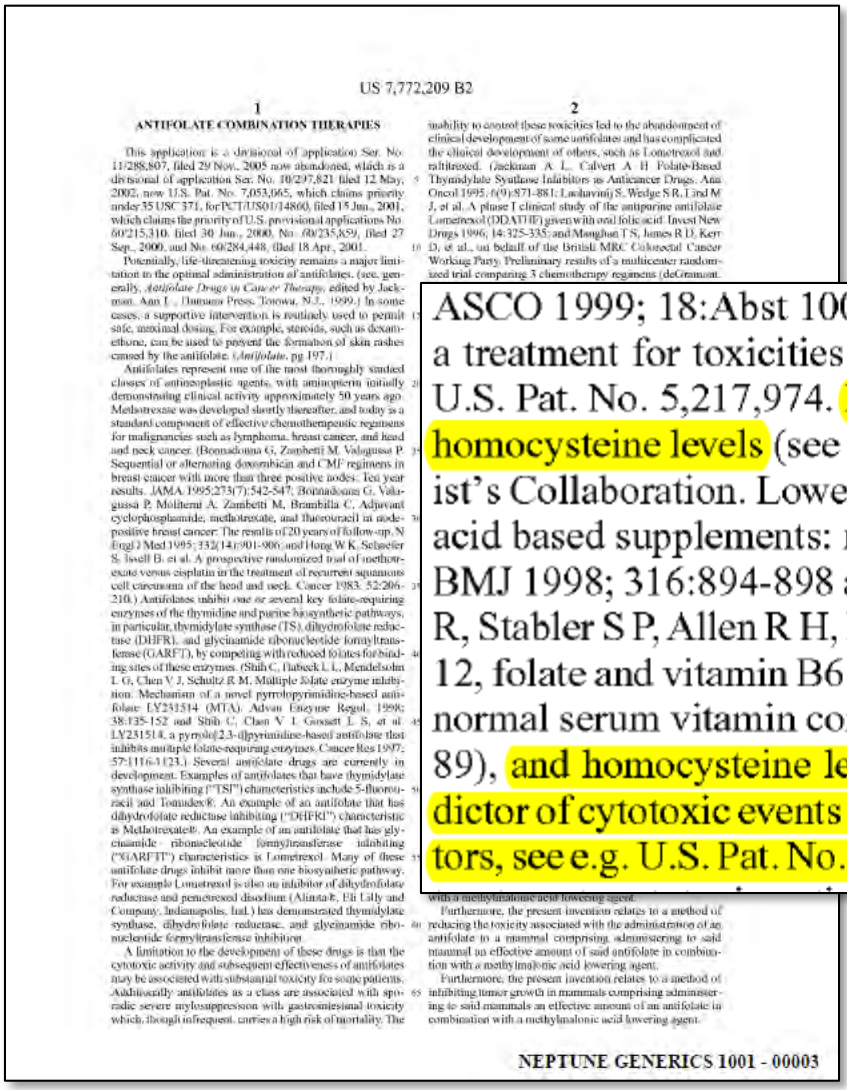
21. The method of claim **12**, **18**, or **19**, wherein the vitamin B12 administration is repeated about every 6 to about every 12 weeks following the administration of vitamin B12 until administration of pemetrexed disodium is discontinued.

22. The method of claim **21** further comprising the administration of cisplatin to the patient.

Source: Ex. 1001 at claims; Paper 1 at *passim*.

’209 Patent

’209 Patent Specification



US 7,772,209 B2

1

ANTIFOLATE COMBINATION THERAPIES

This application is a divisional of application Ser. No. 11/288,807, filed 29 Nov., 2005 now abandoned, which is a divisional of application Ser. No. 10/297,821 filed 12 May, 2002, now U.S. Pat. No. 7,053,065, which claims priority under 35 USC 371, for PCT/US01/14860, filed 18 Jun., 2001, which claims the priority of U.S. provisional applications No. 60/215,310, filed 30 Jun., 2000, No. 60/235,859, filed 27 Sep., 2000, and No. 60/284,448, filed 18 Apr., 2001.

Potentially, life-threatening toxicity remains a major limitation to the optimal administration of antifolates. (see, generally, *Antifolate Drugs in Cancer Therapy*, edited by Jackman, Ann L., Humana Press, Totowa, N.J., 1999.) In some cases, a supportive intervention is routinely used to permit safe, maximal dosing. For example, steroids, such as dexamethone, can be used to prevent the formation of skin rashes caused by the antifolate. (*Antifolate*, pg 197.)

Antifolates represent one of the most thoroughly studied classes of antineoplastic agents, with aminopterin initially demonstrating clinical activity approximately 50 years ago. Methotrexate was developed shortly thereafter, and today is a standard component of effective chemotherapeutic regimens for malignancies such as lymphoma, breast cancer, and head and neck cancer. (Bonnadonna G, Zambetti M, Valagussa P. Sequential or alternating doxorubican and CMF regimens in breast cancer with more than three positive nodes. Ten year results. JAMA 1995;273(7):542-547; Bonnadonna G, Valagussa P, Moliterni A, Zambetti M, Brambilla C. Adjuvant cyclophosphamide, methotrexate, and fluorouracil in node-positive breast cancer: The results of 20 years of follow-up. N Engl J Med 1995; 332(14):901-906; and Hong W K, Schsteifer S, Issell B, et al. A prospective randomized trial of methotrexate versus cisplatin in the treatment of recurrent squamous cell carcinoma of the head and neck. Cancer 1983; 52:206-210.) Antifolates inhibit one or several key folate-requiring enzymes of the thymidine and purine biosynthetic pathways, in particular, thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT), by competing with reduced folates for binding sites of these enzymes. (Shih C, Habeck L L, Mendelsohn L G, Chen V J, Schultz R M. Multiple folate enzyme inhibition: Mechanism of a novel pyrrolopyrimidine-based antifolate LY231514 (MTA). Advan Enzyme Regul. 1998; 38:135-152 and Shih C, Chen V J, Gossett L S, et al. LY231514, a pyrrolo[2,3-d]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. Cancer Res 1997; 57:1116-1123.) Several antifolate drugs are currently in development. Examples of antifolates that have thymidylate synthase inhibiting ("TSI") chameteristics include 5-fluorouracil and Tomudex®. An example of an antifolate that has dihydrofolate reductase inhibiting ("DHFRI") characteristic is Methotrexate®. An example of an antifolate that has glycinamide ribonucleotide formyltransferase inhibiting ("GARFT") characteristics is Lometrexol. Many of these antifolate drugs inhibit more than one biosynthetic pathway. For example Lometrexol is also an inhibitor of dihydrofolate reductase and pemetrexed disodium (Alimta®, Eli Lilly and Company, Indianapolis, Ind.) has demonstrated thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase inhibition.

A limitation to the development of these drugs is that the cytotoxic activity and subsequent effectiveness of antifolates may be associated with substantial toxicity for some patients. Additionally antifolates as a class are associated with sporadic severe myelosuppression with gastrointestinal toxicity which, though infrequent, carries a high risk of mortality. The

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inability to control these toxicities led to the abandonment of clinical development of some antifolates and has complicated the clinical development of others, such as Lometrexol and raltitrexed. (Jackman A. L., Calvert A. H. Folate-Based Thymidylate Synthase Inhibitors as Anticancer Drugs. Ann Oncol 1995; 6(9):871-881; Lacharinij S., Wedge S.R., Lind M. J, et al. A phase I clinical study of the antipurine antifolate Lometrexol (DDATHF) given with oral folic acid. Invest New Drugs 1996; 14:325-335; and Maughan T S, James R D, Kerr D, et al., on behalf of the British MRC Colorectal Cancer Working Party. Preliminary results of a multicenter randomized trial comparing 3 chemotherapy regimens (deGramont

ASCO 1999; 18:Abst 1007.) Initially, folic acid was used as a treatment for toxicities associated with GARFTI see, e.g. U.S. Pat. No. 5,217,974. Folic acid has been shown to lower homocysteine levels (see e.g. Homocysteine Lowering Trialist's Collaboration. Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomized trials. BMJ 1998; 316:894-898 and Naurath H J, Joosten E, Riezler R, Stabler S P, Allen R H, Lindenbaum J. Effects of vitamin B 12, folate and vitamin B6 supplements in elderly people with normal serum vitamin concentrations. Lancet 1995; 346:85-89), and homocysteine levels have been shown to be a predictor of cytotoxic events related to the use of GARFT inhibitors, see e.g. U.S. Pat. No. 5,217,974. However, even with this

with a methylmalonic acid lowering agent.

Furthermore, the present invention relates to a method of reducing the toxicity associated with the administration of an antifolate to a mammal comprising administering to said mammal an effective amount of said antifolate in combination with a methylmalonic acid lowering agent.

Furthermore, the present invention relates to a method of inhibiting tumor growth in mammals comprising administering to said mammals an effective amount of an antifolate in combination with a methylmalonic acid lowering agent.

NEPTUNE GENERICS 1001 - 00003

Source: Ex. 1001 at 2:14-26; Paper 1 at 58; Paper 13 at 3-4.

POSA

POSA Definition

20. I understand that a person of ordinary skill in the art ("POSA") is a hypothetical person presumed to be aware of all pertinent art, understands conventional wisdom in the art, and is a person of ordinary creativity. In this case, a medical doctor with an M.D. degree who has significant experience in treating cancer patients, and a significant understanding of antineoplastic agents, including antifolates and their efficacies, safety, adverse effects, toxicities, etc., is a POSA.

21. A POSA may work as part of a multi-disciplinary team and draw upon not only his or her own skills, but also take advantage of certain specialized skills of others on the team, to solve a given problem. For example, an expert in nutrition, an expert in hematology, a basic scientist with expertise in biochemistry, and a clinician may be part of the team.

Source: Ex. 1025 at ¶¶ 20-21; Paper 1 at 24-25.

PRIOR ART: NIYIKIZA

Prior Art: Niyikiza

Niyikiza

Conclusions: Toxicities resulting from treatment with MTA appear to be predictable from pretreatment homocysteine levels. Elevated baseline homocysteine levels (≥ 10μM) highly correlate with severe hematologic and nonhematologic toxicities following treatment with MTA. Homocysteine was found to be better than albumin at predicting toxicity. These results apply to the tumor types studied. Further studies are underway in patients with renal impairment or patients who received prior cisplatin.

The purpose of this study was to assess the relationship of vitamin metabolites, drug exposure, and other prespecified baseline patient characteristics to toxicity following treatment with MTA.

Methods: Homocysteine (Hcys), cystathionine and methylmalonic acid were measured in 139 phase II patients with tumors of the colon, breast, pancreas, and esophagus at baseline and once each cycle thereafter. Stepwise regression modeling, multivariate analysis of variance, and discriminant analysis were implemented to determine which predictors might correlate with severe toxicity after one course of MTA. Prognostic factors considered were age, gen-

NEPTUNE GENERICS 1008 - 00002

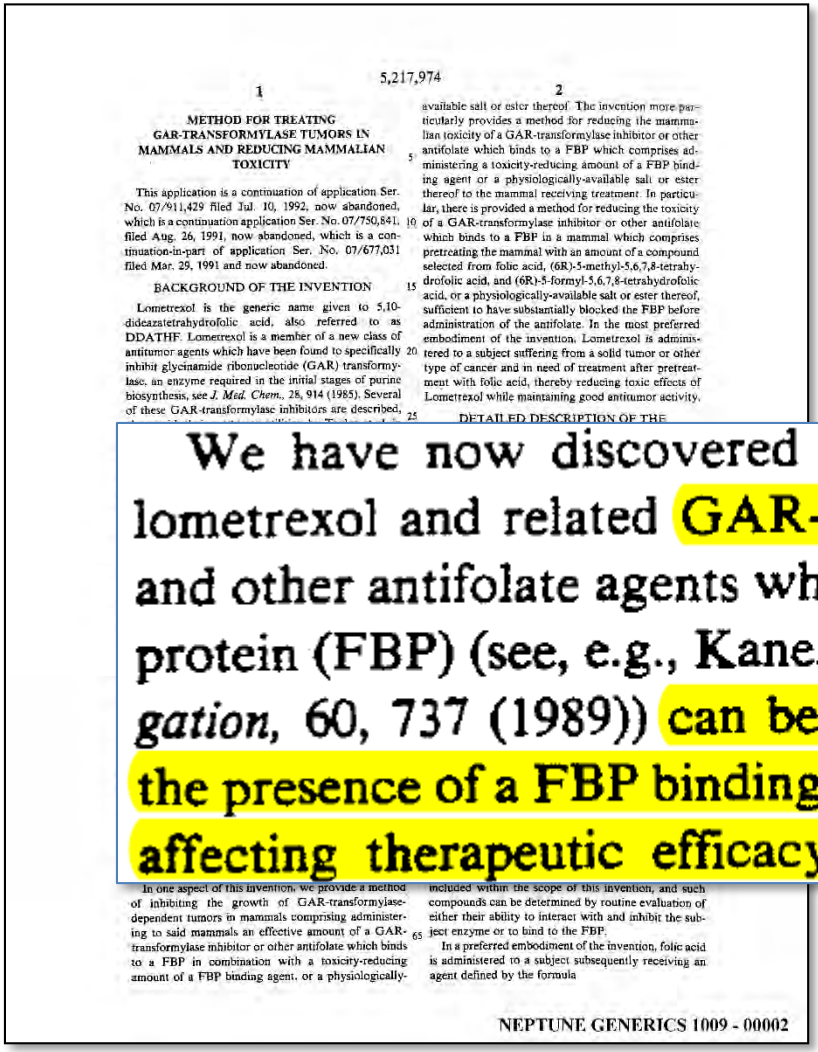
NEPTUNE GENERICS 1008 - 00003

Source: Ex. 1008 at 126-127; Paper 1 at 18, 26-27, 40-41, 49, 59.

PRIOR ART: '974 PATENT

Prior Art: ’974 Patent

’974 Patent



5,217,974

1

METHOD FOR TREATING GAR-TRANSFORMYLASE TUMORS IN MAMMALS AND REDUCING MAMMALIAN TOXICITY

This application is a continuation of application Ser. No. 07/911,429 filed Jul. 10, 1992, now abandoned, which is a continuation application Ser. No. 07/750,841, filed Aug. 26, 1991, now abandoned, which is a continuation-in-part of application Ser. No. 07/677,031 filed Mar. 29, 1991 and now abandoned.

BACKGROUND OF THE INVENTION

Lometrexol is the generic name given to 5,10-dideazatetrahydrofolic acid, also referred to as DDATHF. Lometrexol is a member of a new class of antitumor agents which have been found to specifically inhibit glycinamide ribonucleotide (GAR) transformylase, an enzyme required in the initial stages of purine biosynthesis, see *J. Med. Chem.*, 28, 914 (1985). Several of these GAR-transformylase inhibitors are described,

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available salt or ester thereof. The invention more particularly provides a method for reducing the mammalian toxicity of a GAR-transformylase inhibitor or other antifolate which binds to a FBP which comprises administering a toxicity-reducing amount of a FBP binding agent or a physiologically-available salt or ester thereof to the mammal receiving treatment. In particular, there is provided a method for reducing the toxicity of a GAR-transformylase inhibitor or other antifolate which binds to a FBP in a mammal which comprises pretreating the mammal with an amount of a compound selected from folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, and (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically-available salt or ester thereof, sufficient to have substantially blocked the FBP before administration of the antifolate. In the most preferred embodiment of the invention, Lometrexol is administered to a subject suffering from a solid tumor or other type of cancer and in need of treatment after pretreatment with folic acid, thereby reducing toxic effects of Lometrexol while maintaining good antitumor activity.

DETAILED DESCRIPTION OF THE

In one aspect of this invention, we provide a method of inhibiting the growth of GAR-transformylase-dependent tumors in mammals comprising administering to said mammals an effective amount of a GAR-transformylase inhibitor or other antifolate which binds to a FBP in combination with a toxicity-reducing amount of a FBP binding agent, or a physiologically-

included within the scope of this invention, and such compounds can be determined by routine evaluation of either their ability to interact with and inhibit the subject enzyme or to bind to the FBP.

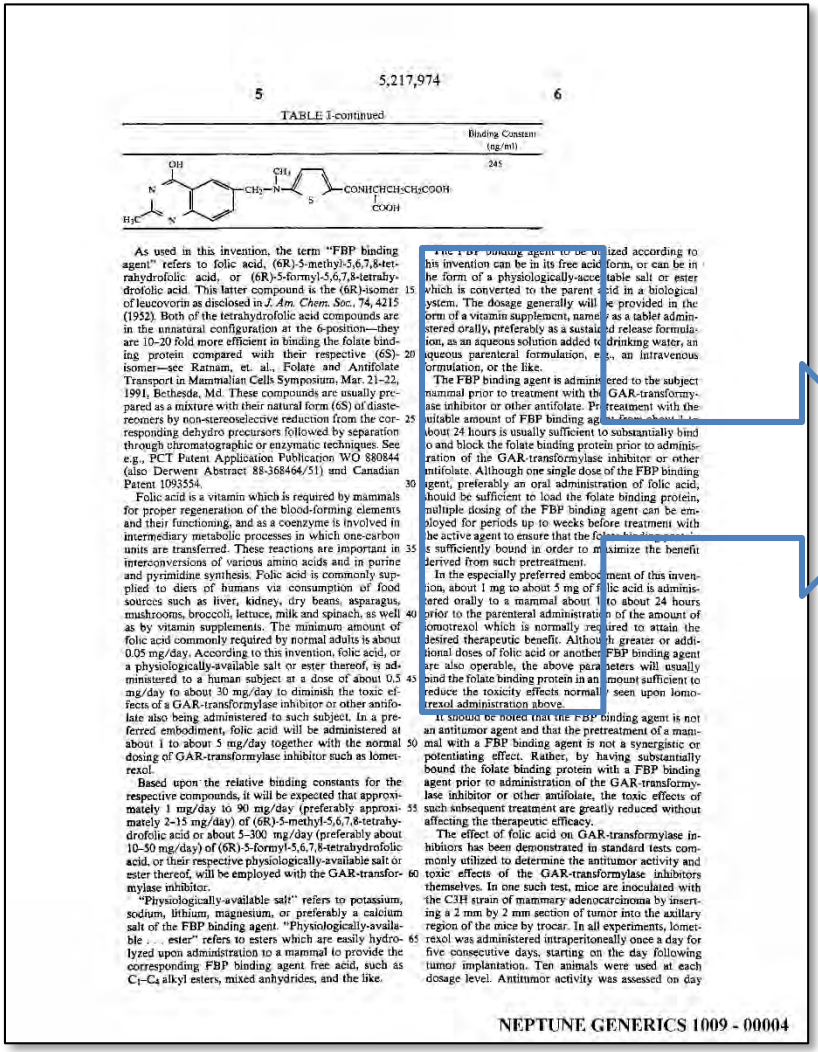
In a preferred embodiment of the invention, folic acid is administered to a subject subsequently receiving an agent defined by the formula

NEPTUNE GENERICS 1009 - 00002

We have now discovered that the toxic effects of lometrexol and related GAR-transformylase inhibitors and other antifolate agents which bind to folate binding protein (FBP) (see, e.g., Kane, et al., *Laboratory Investigation*, 60, 737 (1989)) can be significantly reduced by the presence of a FBP binding agent, without adversely affecting therapeutic efficacy. The present invention

Prior Art: ’974 Patent

’974 Patent



5,217,974

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TABLE I-continued

Binding Constant (ng/ml)

245

As used in this invention, the term "FBP binding agent" refers to folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, or (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid. This latter compound is the (6R)-isomer of leucovorin as disclosed in *J. Am. Chem. Soc.*, 74, 4215 (1952). Both of the tetrahydrofolic acid compounds are in the unnatural configuration at the 6-position—they are 10–20 fold more efficient in binding the folate binding protein compared with their respective (6S)-isomer—see Ratnam, et. al., Folate and Antifolate Transport in Mammalian Cells Symposium, Mar. 21–22, 1991, Bethesda, Md. These compounds are usually prepared as a mixture with their natural form (6S) of diastereomers by non-stereoselective reduction from the corresponding dehydro precursors followed by separation through chromatographic or enzymatic techniques See e.g., PCT Patent Application Publication WO 880844 (also Derwent Abstract 88-368464/51) and Canadian Patent 1093554.

Folic acid is a vitamin which is required by mammals for proper regeneration of the blood-forming elements and their functioning, and as a coenzyme is involved in intermediary metabolic processes in which one-carbon units are transferred. These reactions are important in interconversions of various amino acids and in purine and pyrimidine synthesis. Folic acid is commonly supplied to diets of humans via consumption of food sources such as liver, kidney, dry beans, asparagus, mushrooms, broccoli, lettuce, milk and spinach, as well as by vitamin supplements. The minimum amount of folic acid commonly required by normal adults is about 0.05 mg/day. According to this invention, folic acid, or a physiologically-available salt or ester thereof, is administered to a human subject at a dose of about 0.5 mg/day to about 30 mg/day to diminish the toxic effects of a GAR-transformylase inhibitor or other antifolate also being administered to such subject. In a preferred embodiment, folic acid will be administered at about 1 to about 5 mg/day together with the normal dosing of GAR-transformylase inhibitor such as lometrexol.

Based upon the relative binding constants for the respective compounds, it will be expected that approximately 1 mg/day to 90 mg/day (preferably approximately 2–15 mg/day) of (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid or about 5–300 mg/day (preferably about 10–50 mg/day) of (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or their respective physiologically-available salt or ester thereof, will be employed with the GAR-transformylase inhibitor.

"Physiologically-available salt" refers to potassium, sodium, lithium, magnesium, or preferably a calcium salt of the FBP binding agent. "Physiologically-available . . . ester" refers to esters which are easily hydrolyzed upon administration to a mammal to provide the corresponding FBP binding agent free acid, such as C_1–C_4 alkyl esters, mixed anhydrides, and the like.

The FBP binding agent to be utilized according to this invention can be in its free acid form, or can be in the form of a physiologically-acceptable salt or ester which is converted to the parent acid in a biological system. The dosage generally will be provided in the form of a vitamin supplement, namely as a tablet administered orally, preferably as a sustained release formulation, as an aqueous solution added to drinking water, an aqueous parenteral formulation, e.g., an intravenous formulation, or the like.

The FBP binding agent is administered to the subject mammal prior to treatment with the GAR-transformylase inhibitor or other antifolate. Pretreatment with the suitable amount of FBP binding agent from about 1 to about 24 hours is usually sufficient to substantially bind to and block the folate binding protein prior to administration of the GAR-transformylase inhibitor or other antifolate. Although one single dose of the FBP binding agent, preferably an oral administration of folic acid, should be sufficient to load the folate binding protein, multiple dosing of the FBP binding agent can be employed for periods up to weeks before treatment with the active agent to ensure that the folate binding protein is sufficiently bound in order to maximize the benefit derived from such pretreatment.

In the especially preferred embodiment of this invention, about 1 mg to about 5 mg of folic acid is administered orally to a mammal about 1 to about 24 hours prior to the parenteral administration of the amount of lomotrexol which is normally required to attain the desired therapeutic benefit. Although greater or additional doses of folic acid or another FBP binding agent are also operable, the above parameters will usually bind the folate binding protein in an amount sufficient to reduce the toxicity effects normally seen upon lomotrexol administration above.

It should be noted that the FBP binding agent is not an antitumor agent and that the pretreatment of a mammal with a FBP binding agent is not a synergistic or potentiating effect. Rather, by having substantially bound the folate binding protein with a FBP binding agent prior to administration of the GAR-transformylase inhibitor or other antifolate, the toxic effects of such subsequent treatment are greatly reduced without affecting the therapeutic efficacy.

The effect of folic acid on GAR-transformylase inhibitors has been demonstrated in standard tests commonly utilized to determine the antitumor activity and toxic effects of the GAR-transformylase inhibitors themselves. In one such test, mice are inoculated with the C3H strain of mammary adenocarcinoma by inserting a 2 mm by 2 mm section of tumor into the axillary region of the mice by trocar. In all experiments, lometrexol was administered intraperitoneally once a day for five consecutive days, starting on the day following tumor implantation. Ten animals were used at each dosage level. Antitumor activity was assessed on day

NEPTUNE GENERICS 1009 - 00004

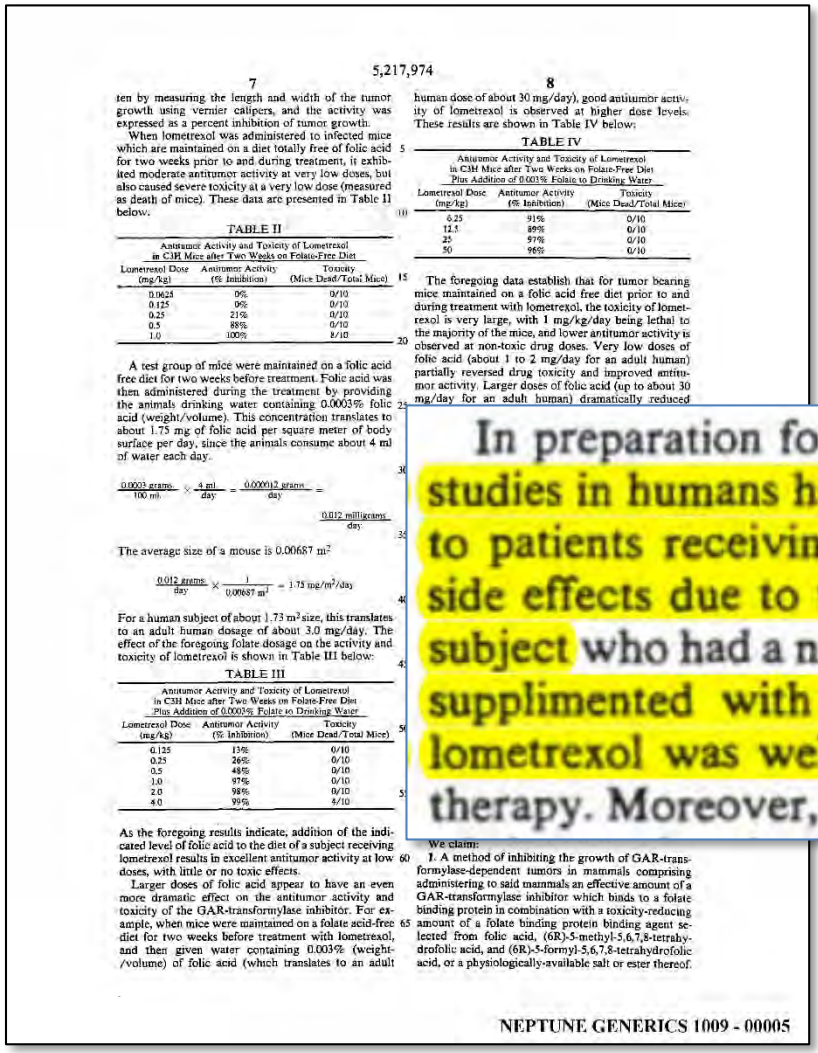
The FBP binding agent is administered to the subject mammal prior to treatment with the GAR-transformylase inhibitor or other antifolate. Pretreatment with the suitable amount of FBP binding agent from about 1 to about 24 hours is usually sufficient to substantially bind to and block the folate binding protein prior to administration of the GAR-transformylase inhibitor or other antifolate. Although one single dose of the FBP binding agent, preferably an oral administration of folic acid, should be sufficient to load the folate binding protein, multiple dosing of the FBP binding agent can be employed for periods up to weeks before treatment with the active agent to ensure that the folate binding protein is sufficiently bound in order to maximize the benefit derived from such pretreatment.

In the especially preferred embodiment of this invention, about 1 mg to about 5 mg of folic acid is administered orally to a mammal about 1 to about 24 hours prior to the parenteral administration of the amount of lomotrexol which is normally required to attain the desired therapeutic benefit. Although greater or additional doses of folic acid or another FBP binding agent are also operable, the above parameters will usually bind the folate binding protein in an amount sufficient to reduce the toxicity effects normally seen upon lomotrexol administration above.

Source: Ex. 1009 at 6:22-36, 6:37-47; Paper 48 at 30.

Prior Art: ’974 Patent

’974 Patent



5,217,974

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ten by measuring the length and width of the tumor growth using vernier calipers, and the activity was expressed as a percent inhibition of tumor growth.

When lometrexol was administered to infected mice which are maintained on a diet totally free of folic acid for two weeks prior to and during treatment, it exhibited moderate antitumor activity at very low doses, but also caused severe toxicity at a very low dose (measured as death of mice). These data are presented in Table II below.

TABLE II

Antitumor Activity and Toxicity of Lometrexol in C3H Mice after Two Weeks on Folate-Free Diet

Lometrexol Dose (mg/kg)	Antitumor Activity (% Inhibition)	Toxicity (Mice Dead/Total Mice)
0.0625	0%	0/10
0.125	0%	0/10
0.25	21%	0/10
0.5	88%	0/10
1.0	100%	8/10

A test group of mice were maintained on a folic acid free diet for two weeks before treatment. Folic acid was then administered during the treatment by providing the animals drinking water containing 0.0003% folic acid (weight/volume). This concentration translates to about 1.75 mg of folic acid per square meter of body surface per day, since the animals consume about 4 ml of water each day.

$$\frac{0.0003 \text{ grams}}{100 \text{ ml}} \times \frac{4 \text{ ml}}{\text{day}} = \frac{0.000012 \text{ grams}}{\text{day}} = \frac{0.012 \text{ milligrams}}{\text{day}}$$

The average size of a mouse is 0.00687 m^2

$$\frac{0.012 \text{ grams}}{\text{day}} \times \frac{1}{0.00687 \text{ m}^2} = 1.75 \text{ mg/m}^2\text{/day}$$

For a human subject of about 1.73 m^2 size, this translates to an adult human dosage of about 3.0 mg/day. The effect of the foregoing folate dosage on the activity and toxicity of lometrexol is shown in Table III below:

TABLE III

Antitumor Activity and Toxicity of Lometrexol in C3H Mice after Two Weeks on Folate-Free Diet Plus Addition of 0.003% Folate to Drinking Water

Lometrexol Dose (mg/kg)	Antitumor Activity (% Inhibition)	Toxicity (Mice Dead/Total Mice)
0.125	13%	0/10
0.25	26%	0/10
0.5	48%	0/10
1.0	97%	0/10
2.0	98%	0/10
4.0	99%	4/10

As the foregoing results indicate, addition of the indicated level of folic acid to the diet of a subject receiving lometrexol results in excellent antitumor activity at low doses, with little or no toxic effects.

Larger doses of folic acid appear to have an even more dramatic effect on the antitumor activity and toxicity of the GAR-transformylase inhibitor. For example, when mice were maintained on a folate acid-free diet for two weeks before treatment with lometrexol, and then given water containing 0.003% (weight-/volume) of folic acid (which translates to an adult human dose of about 30 mg/day), good antitumor activity of lometrexol is observed at higher dose levels. These results are shown in Table IV below:

8

TABLE IV

Antitumor Activity and Toxicity of Lometrexol in C3H Mice after Two Weeks on Folate-Free Diet Plus Addition of 0.003% Folate to Drinking Water

Lometrexol Dose (mg/kg)	Antitumor Activity (% Inhibition)	Toxicity (Mice Dead/Total Mice)
0.25	91%	0/10
12.5	89%	0/10
25	97%	0/10
50	96%	0/10

The foregoing data establish that for tumor bearing mice maintained on a folic acid free diet prior to and during treatment with lometrexol, the toxicity of lometrexol is very large, with 1 mg/kg/day being lethal to the majority of the mice, and lower antitumor activity is observed at non-toxic drug doses. Very low doses of folic acid (about 1 to 2 mg/day for an adult human) partially reversed drug toxicity and improved antitumor activity. Larger doses of folic acid (up to about 30 mg/day for an adult human) dramatically reduced

We claim:

1. A method of inhibiting the growth of GAR-transformylase-dependent tumors in mammals comprising administering to said mammals an effective amount of a GAR-transformylase inhibitor which binds to a folate binding protein in combination with a toxicity-reducing amount of a folate binding protein binding agent selected from folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, and (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically-available salt or ester thereof.

NEPTUNE GENERICS 1009 - 00005

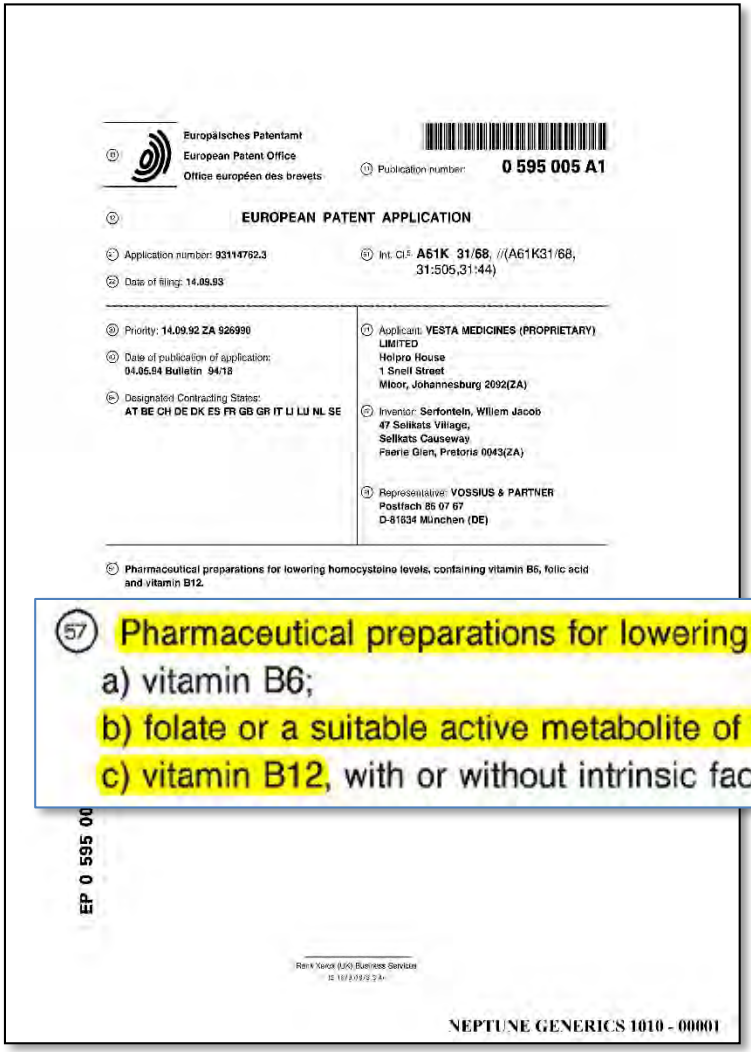
In preparation for the foregoing clinical study, pilot studies in humans have established that folic acid given to patients receiving lometrexol has effected reduced side effects due to the lometrexol. Specifically, in one subject who had a nasalpharyngeal carcinoma, who was supplimented with folic acid at 0.5 to 1.0 mg/day, lometrexol was well tolerated for up to 12 months of therapy. Moreover, this patient has no clinical evidence

Source: Ex. 1009 at 8:49-56; Paper 48 at 30.

PRIOR ART: EP 005

Prior Art: EP 005

EP 005



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(54) Pharmaceutical preparations for lowering homocysteine levels, containing vitamin B6, folic acid and vitamin B12.

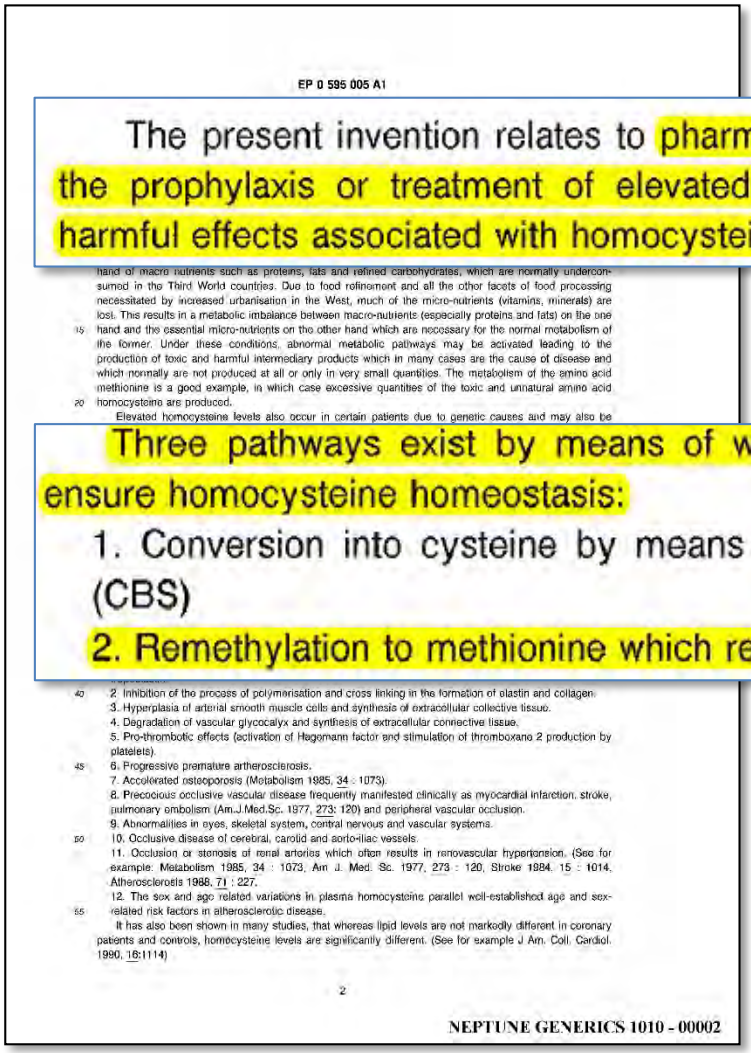
(57) Pharmaceutical preparations for lowering blood and tissue levels of homocysteine are disclosed, comprising:
a) vitamin B6;
b) folate or a suitable active metabolite of folate or a substance which releases folate in vivo;
c) vitamin B12, with or without intrinsic factor

EP 0 595 00

NEPTUNE GENERICS 1010 - 00001

Prior Art: EP 005

EP 005



EP 0 595 005 A1

The present invention relates to pharmaceutical preparations for lowering levels of homocysteine or for the prophylaxis or treatment of elevated levels of homocysteine in patients and for counteracting the harmful effects associated with homocysteine.

hand of macro nutrients such as proteins, fats and refined carbohydrates, which are normally underconsumed in the Third World countries. Due to food refinement and all the other facets of food processing necessitated by increased urbanisation in the West, much of the micro-nutrients (vitamins, minerals) are lost. This results in a metabolic imbalance between macro-nutrients (especially proteins and fats) on the one hand and the essential micro-nutrients on the other hand which are necessary for the normal metabolism of the former. Under these conditions, abnormal metabolic pathways may be activated leading to the production of toxic and harmful intermediary products which in many cases are the cause of disease and which normally are not produced at all or only in very small quantities. The metabolism of the amino acid methionine is a good example, in which case excessive quantities of the toxic and unnatural amino acid homocysteine are produced.

Elevated homocysteine levels also occur in certain patients due to genetic causes and may also be

Three pathways exist by means of which blood and tissue levels of homocysteine are controlled to ensure homocysteine homeostasis:

1. Conversion into cysteine by means of the vitamin B6 dependent enzyme cystathionine β-synthase (CBS)
2. Remethylation to methionine which requires folate (as substrate) and vitamin B12 as co-factor.

2. Inhibition of the process of polymerisation and cross linking in the formation of elastin and collagen.
3. Hyperplasia of arterial smooth muscle cells and synthesis of extracellular collective tissue.
4. Degradation of vascular glycocalyx and synthesis of extracellular connective tissue.
5. Pro-thrombotic effects (activation of Hagemann factor and stimulation of thromboxane 2 production by platelets).
6. Progressive premature atherosclerosis.
7. Accelerated osteoporosis (Metabolism 1985, 34 : 1073).
8. Precocious occlusive vascular disease frequently manifested clinically as myocardial infarction, stroke, pulmonary embolism (Am.J.Med.Sc. 1977, 273: 120) and peripheral vascular occlusion.
9. Abnormalities in eyes, skeletal system, central nervous and vascular systems.
10. Occlusive disease of cerebral, carotid and aorto-iliac vessels.
11. Occlusion or stenosis of renal arteries which often results in renovascular hypertension. (See for example: Metabolism 1985, 34 : 1073; Am J. Med. Sc. 1977, 273 : 120; Stroke 1984, 15 : 1014; Atherosclerosis 1988, 71 : 227.
12. The sex and age related variations in plasma homocysteine parallel well-established age and sex-related risk factors in atherosclerotic disease.

It has also been shown in many studies, that whereas lipid levels are not markedly different in coronary patients and controls, homocysteine levels are significantly different. (See for example J Am. Coll. Cardiol. 1990, 16:1114)

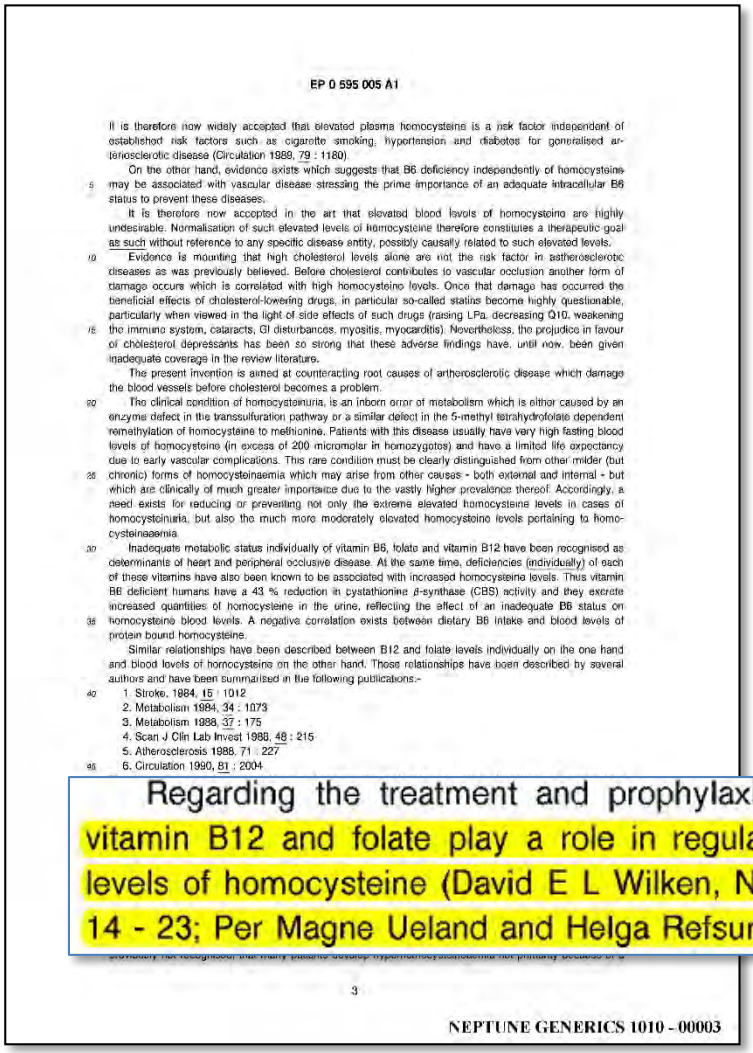
2

NEPTUNE GENERICS 1010 - 00002

Source: Ex. 1010 at 2:1-3; 2:25-29; Paper 1 at 22, 34, 36, 41.

Prior Art: EP 005

EP 005



EP 0 595 005 A1

It is therefore now widely accepted that elevated plasma homocysteine is a risk factor independent of established risk factors such as cigarette smoking, hypertension and diabetes for generalised arteriosclerotic disease (Circulation 1988, 79 : 1180).

On the other hand, evidence exists which suggests that B6 deficiency independently of homocysteine may be associated with vascular disease stressing the prime importance of an adequate intracellular B6 status to prevent these diseases.

It is therefore now accepted in the art that elevated blood levels of homocysteine are highly undesirable. Normalisation of such elevated levels of homocysteine therefore constitutes a therapeutic goal as such without reference to any specific disease entity, possibly causally related to such elevated levels.

Evidence is mounting that high cholesterol levels alone are not the risk factor in artherosclerotic diseases as was previously believed. Before cholesterol contributes to vascular occlusion another form of damage occure which is correlated with high homocysteine levels. Once that damage has occurred the beneficial effects of cholesterol-lowering drugs, in particular so-called statins become highly questionable, particularly when viewed in the light of side effects of such drugs (raising LPa, decreasing Q10, weakening the immune system, cataracts, GI disturbances, myositis, myocarditis). Nevertheless, the prejudice in favour of cholesterol depressants has been so strong that these adverse findings have, until now, been given inadequate coverage in the review literature.

The present invention is aimed at counteracting root causes of artherosclerotic disease which damage the blood vessels before cholesterol becomes a problem.

The clinical condition of homocystinuria, is an inborn error of metabolism which is either caused by an enzyme defect in the transsulfuration pathway or a similar defect in the 5-methyl tetrahydrofolate dependent remethylation of homocysteine to methionine. Patients with this disease usually have very high fasting blood levels of homocysteine (in excess of 200 micromolar in homozygotes) and have a limited life expectancy due to early vascular complications. This rare condition must be clearly distinguished from other milder (but chronic) forms of homocysteinaemia which may arise from other causes - both external and internal - but which are clinically of much greater importance due to the vastly higher prevalence thereof. Accordingly, a need exists for reducing or preventing not only the extreme elevated homocysteine levels in cases of homocystinuria, but also the much more moderately elevated homocysteine levels pertaining to homocysteinaemia.

Inadequate metabolic status individually of vitamin B6, folate and vitamin B12 have been recognised as determinants of heart and peripheral occlusive disease. At the same time, deficiencies (individually) of each of these vitamins have also been known to be associated with increased homocysteine levels. Thus vitamin B6 deficient humans have a 43 % reduction in cystathionine β-synthase (CBS) activity and they excrete increased quantities of homocysteine in the urine, reflecting the effect of an inadequate B6 status on homocysteine blood levels. A negative correlation exists between dietary B6 intake and blood levels of protein bound homocysteine.

Similar relationships have been described between B12 and folate levels individually on the one hand and blood levels of homocysteine on the other hand. Those relationships have been described by several authors and have been summarised in the following publications:-

1. Stroke, 1984, 15 : 1012
2. Metabolism 1984, 34 : 1073
3. Metabolism 1988, 37 : 175
4. Scan J Clin Lab Invest 1988, 48 : 215
5. Atherosclerosis 1988, 71 : 227
6. Circulation 1990, 81 : 2004

3

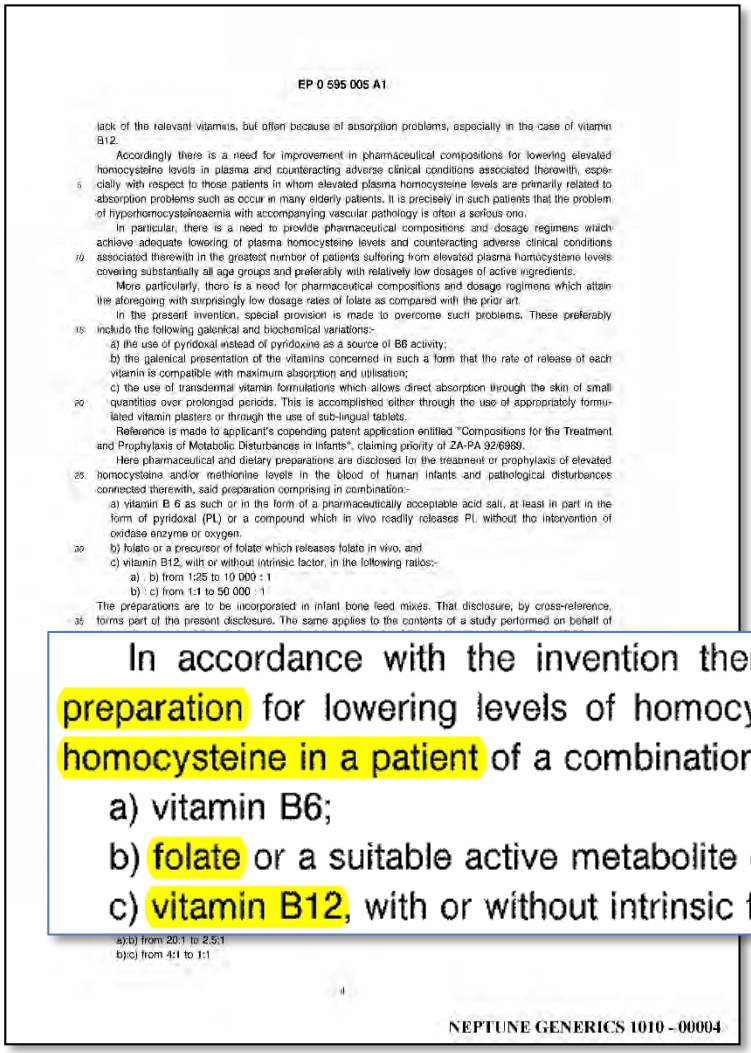
NEPTUNE GENERICS 1010 - 00003

Regarding the treatment and prophylaxis of hyperhomocysteineaemia, it is known that vitamin B6, vitamin B12 and folate play a role in regulating the methionine - homocysteine pathway and controlling levels of homocysteine (David E L Wilken, Nicholas P P Dudman, Haemostasis 1989; 19 (supplement 1) : 14 - 23; Per Magne Ueland and Helga Refsum, J.Lab.Clin.Med. November 1989, 473 - 501. However, it was

Source: Ex. 1010 at 3; Paper 48 at 21-22.

Prior Art: EP 005

EP 005



EP 0 595 005 A1

lack of the relevant vitamins, but often because of absorption problems, especially in the case of vitamin B12.

Accordingly there is a need for improvement in pharmaceutical compositions for lowering elevated homocysteine levels in plasma and counteracting adverse clinical conditions associated therewith, especially with respect to those patients in whom elevated plasma homocysteine levels are primarily related to absorption problems such as occur in many elderly patients. It is precisely in such patients that the problem of hyperhomocysteinaemia with accompanying vascular pathology is often a serious one.

In particular, there is a need to provide pharmaceutical compositions and dosage regimens which achieve adequate lowering of plasma homocysteine levels and counteracting adverse clinical conditions associated therewith in the greatest number of patients suffering from elevated plasma homocysteine levels covering substantially all age groups and preferably with relatively low dosages of active ingredients.

More particularly, there is a need for pharmaceutical compositions and dosage regimens which attain the aforegoing with surprisingly low dosage rates of folate as compared with the prior art.

In the present invention, special provision is made to overcome such problems. These preferably include the following galenical and biochemical variations:-

a) the use of pyridoxal instead of pyridoxine as a source of B6 activity;

b) the galenical presentation of the vitamins concerned in such a form that the rate of release of each vitamin is compatible with maximum absorption and utilisation;

c) the use of transdermal vitamin formulations which allows direct absorption through the skin of small quantities over prolonged periods. This is accomplished either through the use of appropriately formulated vitamin plasters or through the use of sub-lingual tablets.

Reference is made to applicant's copending patent application entitled "Compositions for the Treatment and Prophylaxis of Metabolic Disturbances in Infants", claiming priority of ZA-PA 92/6989.

Here pharmaceutical and dietary preparations are disclosed for the treatment or prophylaxis of elevated homocysteine and/or methionine levels in the blood of human infants and pathological disturbances connected therewith, said preparation comprising in combination:-

a) vitamin B 6 as such or in the form of a pharmaceutically acceptable acid salt, at least in part in the form of pyridoxal (PL) or a compound which in vivo readily releases PL without the intervention of oxidase enzyme or oxygen.

b) folate or a precursor of folate which releases folate in vivo, and

c) vitamin B12, with or without intrinsic factor, in the following ratios:-

a) : b) from 1:25 to 10 000 : 1

b) : c) from 1:1 to 50 000 : 1

The preparations are to be incorporated in infant bone feed mixes. That disclosure, by cross-reference, forms part of the present disclosure. The same applies to the contents of a study performed on behalf of

a):b) from 20:1 to 2.5:1

b):c) from 4:1 to 1:1

4

NEPTUNE GENERICS 1010 - 00004

In accordance with the invention there is provided the use in the manufacture of a pharmaceutical preparation for lowering levels of homocysteine or for the prophylaxis or treatment of elevated levels of homocysteine in a patient of a combination which comprises

a) vitamin B6;

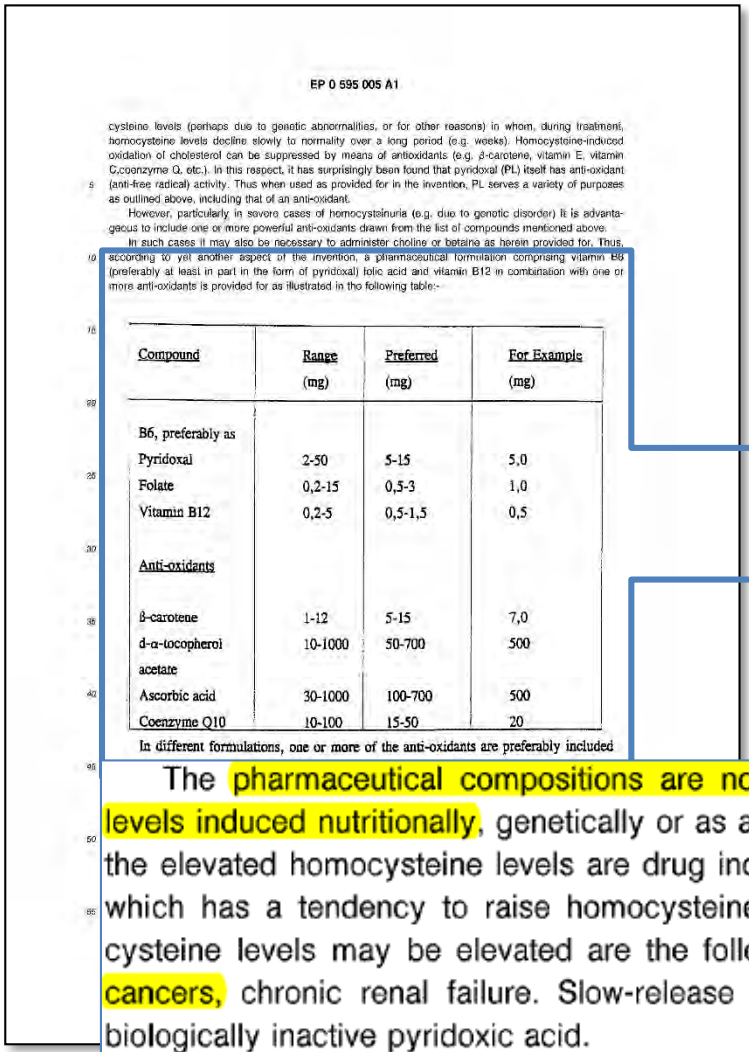
b) folate or a suitable active metabolite of folate or a substance which releases folate in vivo;

c) vitamin B12, with or without intrinsic factor.

Source: Ex. 1010 at 4; Paper 48 at 21-22.

Prior Art: EP 005

EP 005



EP 0 595 005 A1

cysteine levels (perhaps due to genetic abnormalities, or for other reasons) in whom, during treatment, homocysteine levels decline slowly to normality over a long period (e.g. weeks). Homocysteine-induced oxidation of cholesterol can be suppressed by means of antioxidants (e.g. β-carotene, vitamin E, vitamin C,coenzyme Q, etc.). In this respect, it has surprisingly been found that pyridoxal (PL) itself has anti-oxidant (anti-free radical) activity. Thus when used as provided for in the invention, PL serves a variety of purposes as outlined above, including that of an anti-oxidant.

However, particularly in severe cases of homocystsinuria (e.g. due to genetic disorder) it is advantageous to include one or more powerful anti-oxidants drawn from the list of compounds mentioned above.

In such cases it may also be necessary to administer choline or betaine as herein provided for. Thus, according to yet another aspect of the invention, a pharmaceutical formulation comprising vitamin B6 (preferably at least in part in the form of pyridoxal) folic acid and vitamin B12 in combination with one or more anti-oxidants is provided for as illustrated in the following table:-

Compound	Range (mg)	Preferred (mg)	For Example (mg)
B6, preferably as Pyridoxal	2-50	5-15	5,0
Folate	0,2-15	0,5-3	1,0
Vitamin B12	0,2-5	0,5-1,5	0,5
Anti-oxidants			
ß-carotene	1-12	5-15	7,0
d-α-tocopherol acetate	10-1000	50-700	500
Ascorbic acid	30-1000	100-700	500
Coenzyme Q10	10-100	15-50	20

In different formulations, one or more of the anti-oxidants are preferably included

according to yet another aspect of the invention, a pharmaceutical formulation comprising vitamin B6 (preferably at least in part in the form of pyridoxal) folic acid and vitamin B12 in combination with one or more anti-oxidants is provided for as illustrated in the following table:-

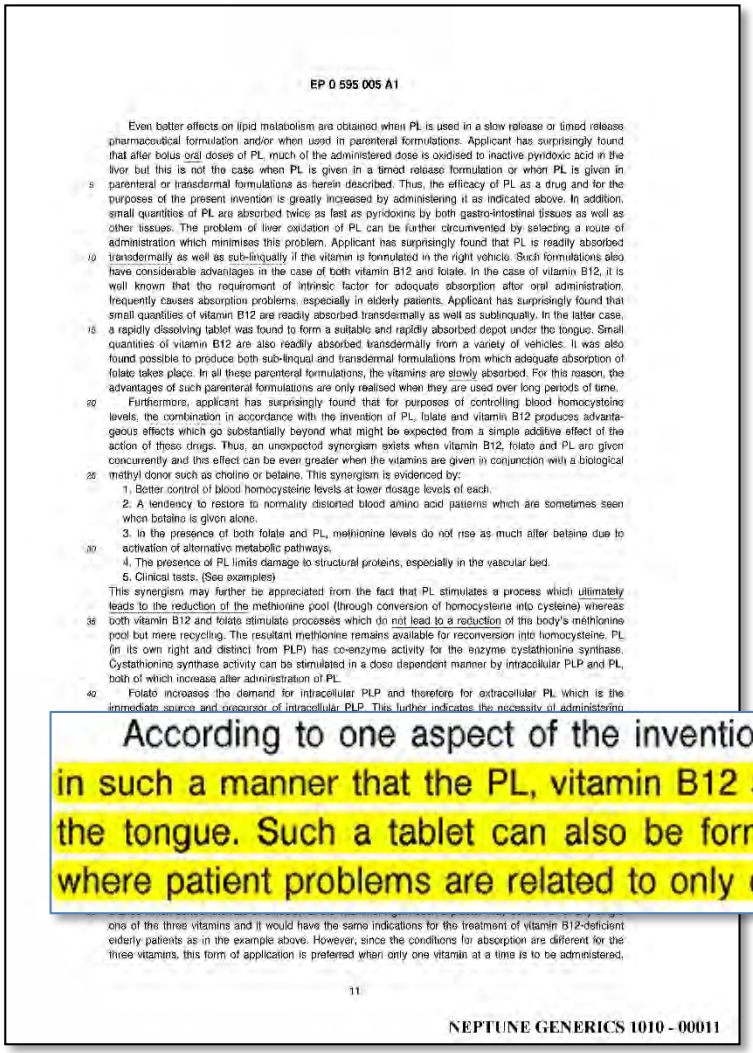
Compound	Range (mg)	Preferred (mg)	For Example (mg)
B6, preferably as Pyridoxal	2-50	5-15	5,0
Folate	0,2-15	0,5-3	1,0
Vitamin B12	0,2-5	0,5-1,5	0,5
Anti-oxidants			
ß-carotene	1-12	5-15	7,0
d-α-tocopherol acetate	10-1000	50-700	500
Ascorbic acid	30-1000	100-700	500
Coenzyme Q10	10-100	15-50	20

The pharmaceutical compositions are not only to be used in the treatment of raised homocysteine levels induced nutritionally, genetically or as a result of a variety of diseases, but also in those cases where the elevated homocysteine levels are drug induced or in combination with a B6 or folate antagonistic drug, which has a tendency to raise homocysteine levels. Examples of other situations in which blood homocysteine levels may be elevated are the following: post-menopausal women, liver failure, leukemia, other cancers, chronic renal failure. Slow-release formulation of PL prevents excessive liver oxidation to the biologically inactive pyridoxic acid.

Source: Ex. 1010 at 9; Paper 48 at 22.

Prior Art: EP 005

EP 005



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Even better effects on lipid metabolism are obtained when PL is used in a slow release or timed release pharmaceutical formulation and/or when used in parenteral formulations. Applicant has surprisingly found that after bolus oral doses of PL much of the administered dose is oxidised to inactive pyridoxic acid in the liver but this is not the case when PL is given in a timed release formulation or when PL is given in parenteral or transdermal formulations as herein described. Thus, the efficacy of PL as a drug and for the purposes of the present invention is greatly increased by administering it as indicated above. In addition, small quantities of PL are absorbed twice as fast as pyridoxine by both gastro-intestinal tissues as well as other tissues. The problem of liver oxidation of PL can be further circumvented by selecting a route of administration which minimises this problem. Applicant has surprisingly found that PL is readily absorbed transdermally as well as sub-linqually if the vitamin is formulated in the right vehicle. Such formulations also have considerable advantages in the case of both vitamin B12 and folate. In the case of vitamin B12, it is well known that the requirement of intrinsic factor for adequate absorption after oral administration frequently causes absorption problems, especially in elderly patients. Applicant has surprisingly found that small quantities of vitamin B12 are readily absorbed transdermally as well as sublinqually. In the latter case, a rapidly dissolving tablet was found to form a suitable and rapidly absorbed depot under the tongue. Small quantities of vitamin B12 are also readily absorbed transdermally from a variety of vehicles. It was also found possible to produce both sub-linqual and transdermal formulations from which adequate absorption of folate takes place. In all these parenteral formulations, the vitamins are slowly absorbed. For this reason, the advantages of such parenteral formulations are only realised when they are used over long periods of time.

Furthermore, applicant has surprisingly found that for purposes of controlling blood homocysteine levels, the combination in accordance with the invention of PL, folate and vitamin B12 produces advantageous effects which go substantially beyond what might be expected from a simple additive effect of the action of these drugs. Thus, an unexpected synergism exists when vitamin B12, folate and PL are given concurrently and this effect can be even greater when the vitamins are given in conjunction with a biological methyl donor such as choline or betaine. This synergism is evidenced by:

1. Better control of blood homocysteine levels at lower dosage levels of each.
2. A tendency to restore to normality distorted blood amino acid patterns which are sometimes seen when betaine is given alone.
3. In the presence of both folate and PL, methionine levels do not rise as much after betaine due to activation of alternative metabolic pathways.
4. The presence of PL limits damage to structural proteins, especially in the vascular bed.
5. Clinical tests. (See examples)

This synergism may further be appreciated from the fact that PL stimulates a process which ultimately leads to the reduction of the methionine pool (through conversion of homocysteine into cysteine) whereas both vitamin B12 and folate stimulate processes which do not lead to a reduction of the body's methionine pool but mere recycling. The resultant methionine remains available for reconversion into homocysteine. PL (in its own right and distinct from PLP) has co-enzyme activity for the enzyme cystathionine synthase. Cystathionine synthase activity can be stimulated in a dose dependent manner by intracellular PLP and PL, both of which increase after administration of PL.

Folate increases the demand for intracellular PLP and therefore for extracellular PL which is the immediate source and precursor of intracellular PLP. This further indicates the necessity of administering

one of the three vitamins and it would have the same indications for the treatment of vitamin B12-deficient elderly patients as in the example above. However, since the conditions for absorption are different for the three vitamins, this form of application is preferred when only one vitamin at a time is to be administered.

11

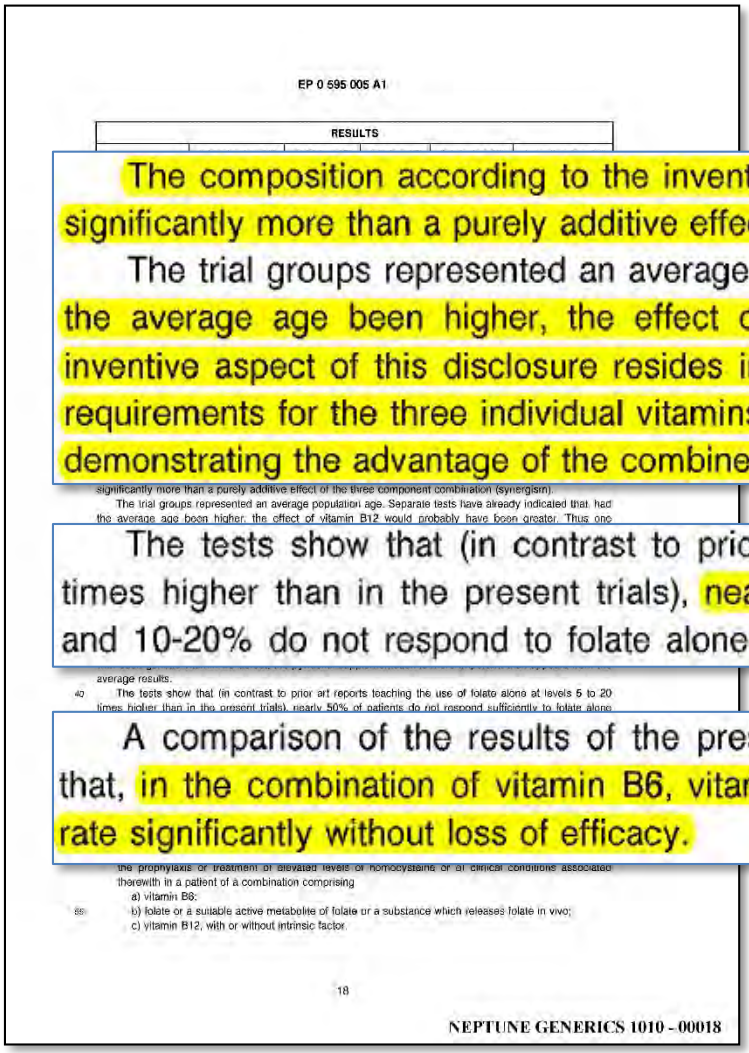
NEPTUNE GENERICS 1010 - 00011

According to one aspect of the invention, a sub-linqual tablet (preferably suitably buffered) is produced in such a manner that the PL, vitamin B12 and folate components are liberated and absorbed mainly under the tongue. Such a tablet can also be formulated to contain all or any one of the three vitamins for use where patient problems are related to only one of these vitamins. A typical example would be the treatment

Source: Ex. 1010 at 11:43-46; Paper 1 at 46-47.

Prior Art: EP 005

EP 005



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RESULTS

The composition according to the invention is nearly twice as effective as folate alone. This indicates a significantly more than a purely additive effect of the three component combination (synergism).

The trial groups represented an average population age. Separate tests have already indicated that, had the average age been higher, the effect of vitamin B12 would probably have been greater. Thus one inventive aspect of this disclosure resides in the clear recognition that different age groups have different requirements for the three individual vitamins in relation to their effects on plasma homocysteine, thus again demonstrating the advantage of the combined use of the three vitamins.

The tests show that (in contrast to prior art reports teaching the use of folate alone at levels 5 to 20 times higher than in the present trials), nearly 50% of patients do not respond sufficiently to folate alone and 10-20% do not respond to folate alone at all, (not even if the folate dosage rate is greatly increased).

A comparison of the results of the present trial with those of the trial according to Example 1 shows that, in the combination of vitamin B6, vitamin B12 and folate it was possible to reduce the folate dosage rate significantly without loss of efficacy.

the prophylaxis or treatment of elevated levels of homocysteine or of clinical conditions associated therewith in a patient of a combination comprising

a) vitamin B6;

b) folate or a suitable active metabolite of folate or a substance which releases folate in vivo;

c) vitamin B12, with or without intrinsic factor.

18

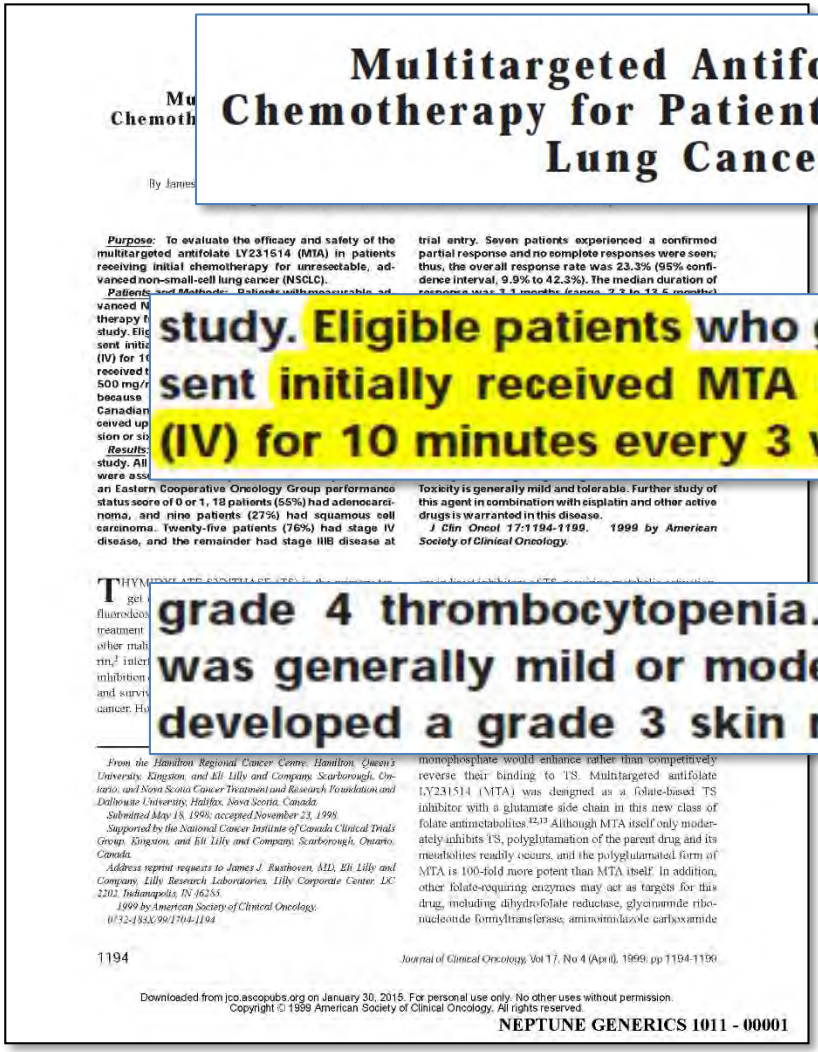
NEPTUNE GENERICS 1010 - 00018

Source: Ex. 1010 at 18; Paper 48 at 21.

PRIOR ART: RUSTHOVEN

Prior Art: Rusthoven

Rusthoven



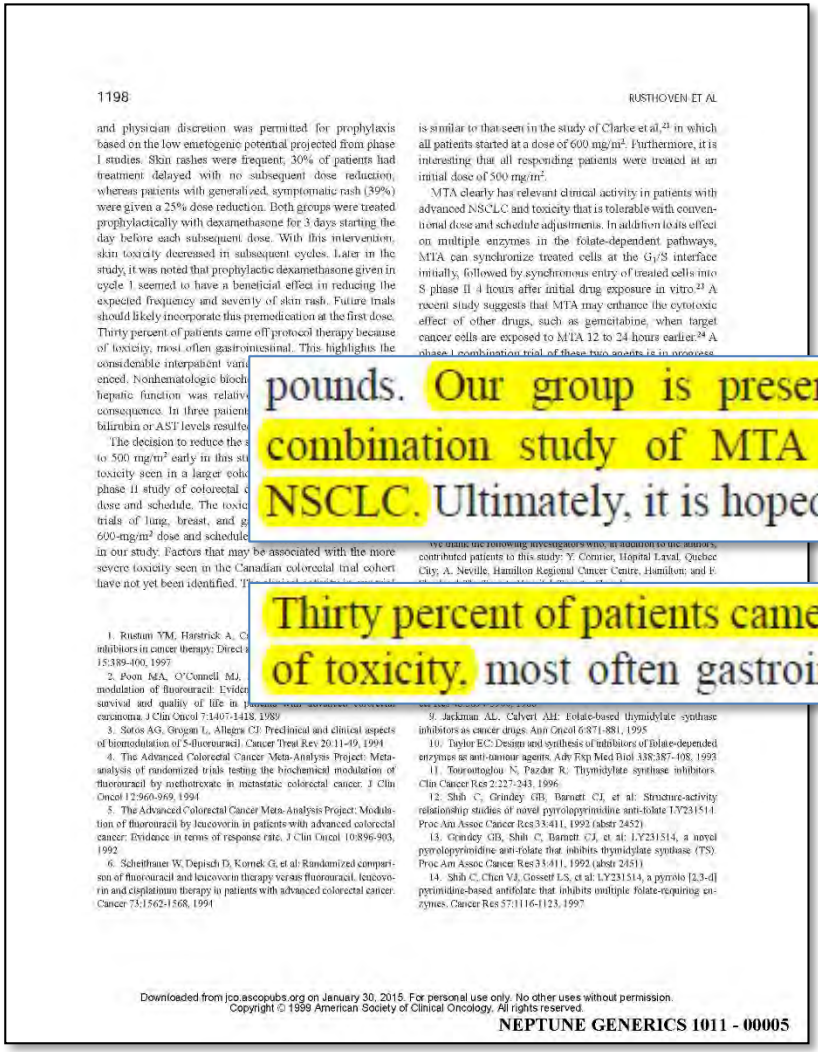
Multitargeted Antifolate LY231514 as First-Line Chemotherapy for Patients With Advanced Non–Small-Cell Lung Cancer: A Phase II Study

study. Eligible patients who gave written informed consent initially received MTA 600 mg/m² intravenously (IV) for 10 minutes every 3 weeks. After three patients

grade 4 thrombocytopenia. Nonhematologic toxicity was generally mild or moderate, but 39% of patients developed a grade 3 skin rash. Most other toxicities

Prior Art: Rusthoven

Rusthoven



1198 RUSTHOVEN ET AL

and physician discretion was permitted for prophylaxis based on the low emetogenic potential projected from phase I studies. Skin rashes were frequent; 30% of patients had treatment delayed with no subsequent dose reduction, whereas patients with generalized, symptomatic rash (39%) were given a 25% dose reduction. Both groups were treated prophylactically with dexamethasone for 3 days starting the day before each subsequent dose. With this intervention, skin toxicity decreased in subsequent cycles. Later in the study, it was noted that prophylactic dexamethasone given in cycle 1 seemed to have a beneficial effect in reducing the expected frequency and severity of skin rash. Future trials should likely incorporate this premedication at the first dose. Thirty percent of patients came off protocol therapy because of toxicity, most often gastrointestinal. This highlights the considerable interpatient vari
enced. Nonhematologic bioch
hepatic function was relativ
consequence. In three patient
bilirubin or AST levels resulte
The decision to reduce the s
to 500 mg/m² early in this st
toxicity seen in a larger cohc
phase II study of colorectal c
dose and schedule. The toxic
trials of lung, breast, and g
600-mg/m² dose and schedule
in our study. Factors that may be associated with the more severe toxicity seen in the Canadian colorectal trial cohort have not yet been identified.

is similar to that seen in the study of Clarke et al,[23] in which all patients started at a dose of 600 mg/m². Furthermore, it is interesting that all responding patients were treated at an initial dose of 500 mg/m².

MTA clearly has relevant clinical activity in patients with advanced NSCLC and toxicity that is tolerable with conventional dose and schedule adjustments. In addition to its effect on multiple enzymes in the folate-dependent pathways, MTA can synchronize treated cells at the G1/S interface initially, followed by synchronous entry of treated cells into S phase 8 hours after initial drug exposure in vitro.[23] A recent study suggests that MTA may enhance the cytotoxic effect of other drugs, such as gemcitabine, when target cancer cells are exposed to MTA 12 to 24 hours earlier.[24] A phase I combination trial of these two agents is in progress

pounds. Our group is presently conducting a phase II combination study of MTA and cisplatin in advanced NSCLC. Ultimately, it is hoped that MTA may contribute to

Thirty percent of patients came off protocol therapy because of toxicity, most often gastrointestinal. This highlights the

contributed patients to this study: Y. Cormier, Hôpital Laval, Quebec City; A. Neville, Hamilton Regional Cancer Centre, Hamilton; and E.

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Source: Ex. 1011 at 1198; Paper 48 at 12.

FA, MTA, AND THE THERAPEUTIC INDEX

FA, MTA and the Therapeutic Index

Rusthoven

hours.[18] Early studies have suggested that dietary supplementation with folic acid may improve the therapeutic index by reducing toxicity in mice.

Hammond I

mg/m^2. As preclinical evaluations indicate that FA supplementation increases the therapeutic index of MTA, this study was initiated to determine if FA supplementation permits significant dose-escalation above the recommended phase II dose of MTA alone. Vitamin metabolites were measured to determine their

Conclusions: FA supplementation appears to permit MTA dose escalation by ameliorating toxicity. Heavily- and minimally-pretreated pts tolerate MTA at

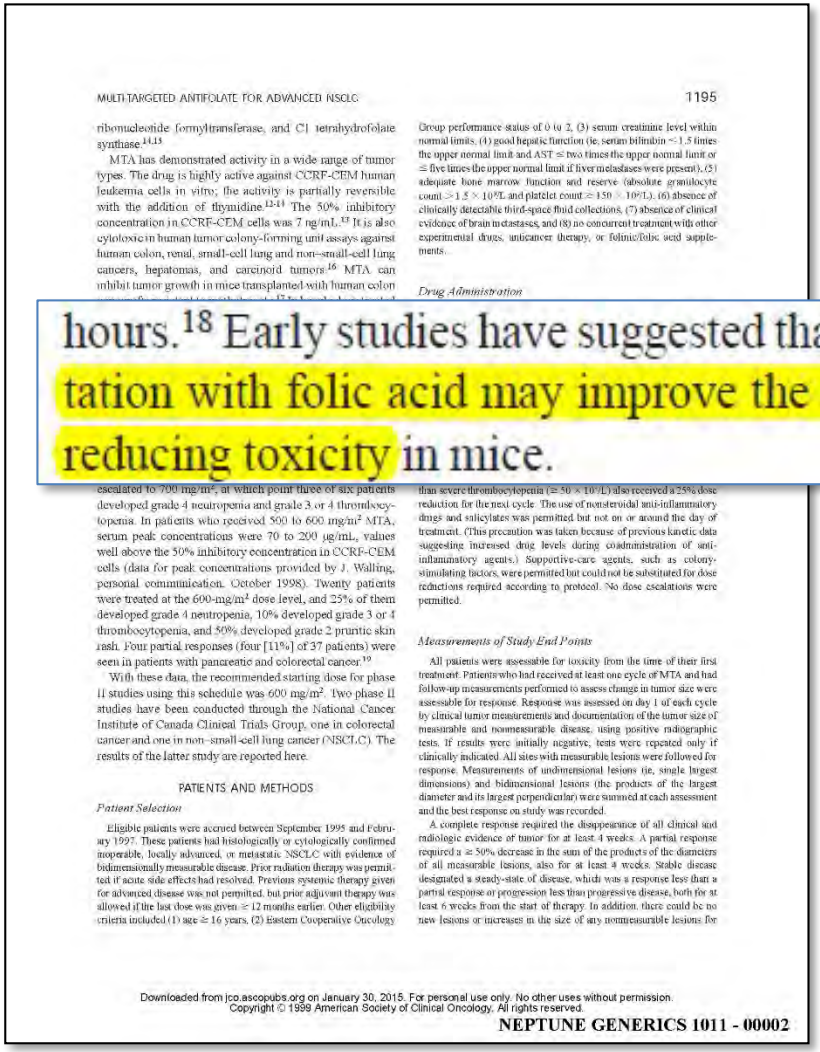
Hammond II

500–600 mg/m^2. Since preclinical studies indicated that folic acid supplementation increases the therapeutic 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 toxic 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

respectively. These results indicate that folic acid supplementation appears to permit MTA dose escalation.

FA, MTA and the Therapeutic Index

Rusthoven



MULTITARGETED ANTIFOLATE FOR ADVANCED NSCLC 1195

ribonucleotide formyltransferase, and C1 tetrahydrofolate synthase.[14,15]

MTA has demonstrated activity in a wide range of tumor types. The drug is highly active against CCRF-CEM human leukemia cells in vitro; the activity is partially reversible with the addition of thymidine.[12-14] The 50% inhibitory concentration in CCRF-CEM cells was 7 ng/mL.[13] It is also cytotoxic in human tumor colony-forming unit assays against human colon, renal, small-cell lung and non–small-cell lung cancers, hepatomas, and carcinoid tumors.[16] MTA can inhibit tumor growth in mice transplanted with human colon

hours.[18] Early studies have suggested that dietary supplementation with folic acid may improve the therapeutic index by reducing toxicity in mice.

escalated to 700 mg/m², at which point three of six patients developed grade 4 neutropenia and grade 3 or 4 thrombocytopenia. In patients who received 500 to 600 mg/m² MTA, serum peak concentrations were 70 to 200 μg/mL, values well above the 50% inhibitory concentration in CCRF-CEM cells (data for peak concentrations provided by J. Walling, personal communication, October 1998). Twenty patients were treated at the 600-mg/m² dose level, and 25% of them developed grade 4 neutropenia, 10% developed grade 3 or 4 thrombocytopenia, and 50% developed grade 2 pruritic skin rash. Four partial responses (four [11%] of 37 patients) were seen in patients with pancreatic and colorectal cancer.[19]

With these data, the recommended starting dose for phase II studies using this schedule was 600 mg/m². Two phase II studies have been conducted through the National Cancer Institute of Canada Clinical Trials Group, one in colorectal cancer and one in non–small-cell lung cancer (NSCLC). The results of the latter study are reported here.

PATIENTS AND METHODS

Patient Selection

Eligible patients were accrued between September 1995 and February 1997. These patients had histologically or cytologically confirmed inoperable, locally advanced, or metastatic NSCLC with evidence of bidimensionally measurable disease. Prior radiation therapy was permitted if acute side effects had resolved. Previous systemic therapy given for advanced disease was not permitted, but prior adjuvant therapy was allowed if the last dose was given ≥ 12 months earlier. Other eligibility criteria included (1) age ≥ 16 years, (2) Eastern Cooperative Oncology Group performance status of 0 to 2, (3) serum creatinine level within normal limits, (4) good hepatic function (ie, serum bilirubin ≤ 1.5 times the upper normal limit and AST ≤ two times the upper normal limit or ≤ five times the upper normal limit if liver metastases were present), (5) adequate bone marrow function and reserve (absolute granulocyte count ≥ 1.5×10^9/L and platelet count ≥ 150×10^9/L), (6) absence of clinically detectable third-space fluid collections, (7) absence of clinical evidence of brain metastases, and (8) no concurrent treatment with other experimental drugs, anticancer therapy, or folinic/folic acid supplements.

Drug Administration

than severe thrombocytopenia (≥ 50×10^9/L) also received a 25% dose reduction for the next cycle. The use of nonsteroidal anti-inflammatory drugs and salicylates was permitted but not on or around the day of treatment. (This precaution was taken because of previous kinetic data suggesting increased drug levels during coadministration of anti-inflammatory agents.) Supportive-care agents, such as colony-stimulating factors, were permitted but could not be substituted for dose reductions required according to protocol. No dose escalations were permitted.

Measurements of Study End Points

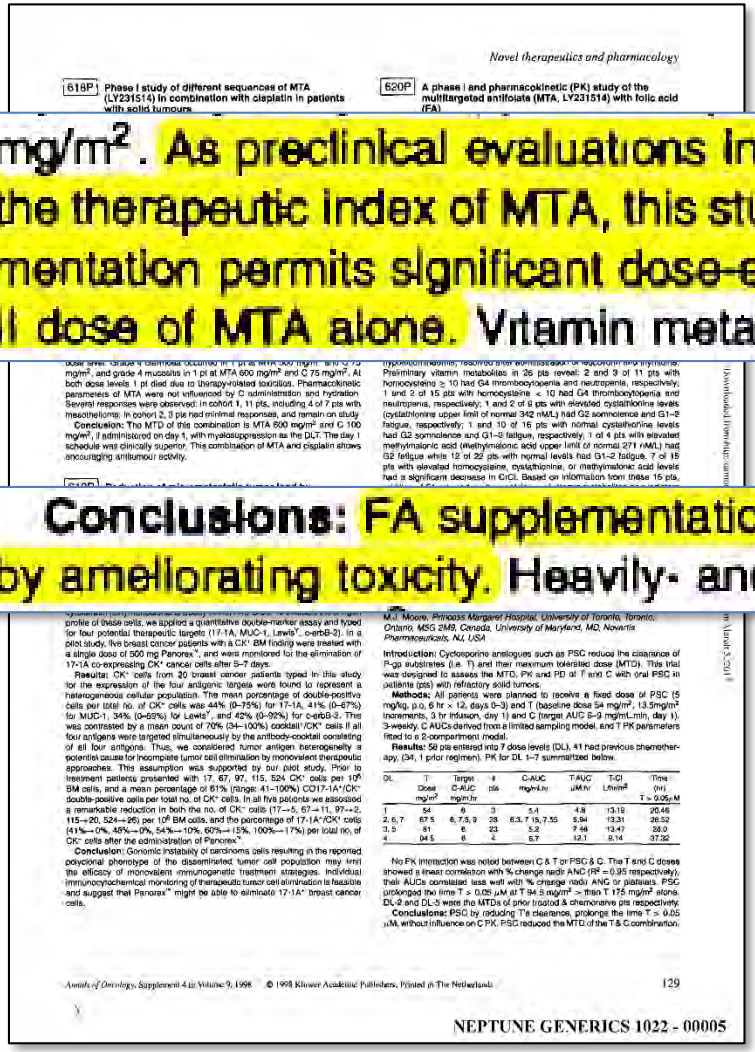
All patients were assessable for toxicity from the time of their first treatment. Patients who had received at least one cycle of MTA and had follow-up measurements performed to assess change in tumor size were assessable for response. Response was assessed on day 1 of each cycle by clinical tumor measurements and documentation of the tumor size of measurable and nonmeasurable disease, using positive radiographic tests. If results were initially negative, tests were repeated only if clinically indicated. All sites with measurable lesions were followed for response. Measurements of unidimensional lesions (ie, single largest dimensions) and bidimensional lesions (the products of the largest diameter and its largest perpendicular) were summed at each assessment and the best response on study was recorded.

A complete response required the disappearance of all clinical and radiologic evidence of tumor for at least 4 weeks. A partial response required a ≥ 50% decrease in the sum of the products of the diameters of all measurable lesions, also for at least 4 weeks. Stable disease designated a steady-state of disease, which was a response less than a partial response or progression less than progressive disease, both for at least 6 weeks from the start of therapy. In addition, there could be no new lesions or increases in the size of any nonmeasurable lesions for

Source: Ex. 1011 at 1195; Paper 48 at 14-15, 22.

FA, MTA and the Therapeutic Index

Hammond



Novel therapeutics and pharmacology

618P Phase I study of different sequences of MTA (LY231514) in combination with cisplatin in patients with solid tumours

620P A phase I and pharmacokinetic (PK) study of the multitargeted antifolate (MTA, LY231514) with folic acid (FA)

mg/m^2. As preclinical evaluations indicate that FA supplementation increases the therapeutic index of MTA, this study was initiated to determine if FA supplementation permits significant dose-escalation above the recommended phase II dose of MTA alone. Vitamin metabolites were measured to determine their

Conclusions: FA supplementation appears to permit MTA dose escalation by ameliorating toxicity. Heavily- and minimally-pretreated pts tolerate MTA at

M.J. Moore, *Princess Margaret Hospital, University of Toronto, Toronto, Ontario, M5G 2M9, Canada, University of Maryland, MD, Novartis Pharmaceuticals, NJ, USA*

Introduction: Cyclosporine analogues such as PSC reduce the clearance of P-gp substrates (i.e. T) and their maximum tolerated dose (MTD). This trial was designed to assess the MTD, PK and PD of T and C with oral PSC in patients (pts) with refractory solid tumors.

Methods: All patients were planned to receive a fixed dose of PSC (5 mg/kg, p.o, 6 hr × 12, days 0–3) and T (baseline dose 54 mg/m^2, 13.5mg/m^2 increments, 3 hr infusion, day 1) and C (target AUC 8–9 mg/mL·min, day 1), 3-weekly. C AUCs derived from a limited sampling model, and T PK parameters fitted to a 2-compartment model.

Results: 58 pts entered into 7 dose levels (DL), 41 had previous chemotherapy, (34, 1 prior regimen). PK for DL 1–7 summarized below.

DL	T Dose mg/m^2	Target C-AUC mg/mL·hr	# pts	C-AUC mg/mL·hr	T·AUC µM·hr	T·Cl L/hr/m^2	Time (hr) T > 0.05 µM
1	54	8	3	5.4	4.8	13.19	20.46
2, 5, 7	67.5	6, 7.5, 9	28	6.3, 7.15, 7.55	5.84	13.31	26.52
3, 5	81	6	23	5.2	7.48	13.47	33.0
4	94.5	6	4	5.7	12.1	9.14	37.32

No PK interaction was noted between C & T or PSC & C. The T and C doses showed a linear correlation with % change nadir ANC (R^2 = 0.95 respectively); their AUCs correlated less well with % change nadir ANC or platelets. PSC prolonged the time T > 0.05 µM at T 94.5 mg/m^2 > than T 175 mg/m^2 alone. DL-2 and DL-3 were the MTDs of prior treated & chemonaive pts respectively.

Conclusions: PSC by reducing T's clearance, prolongs the time T > 0.05 µM, without influence on C PK. PSC reduced the MTD of the T & C combination.

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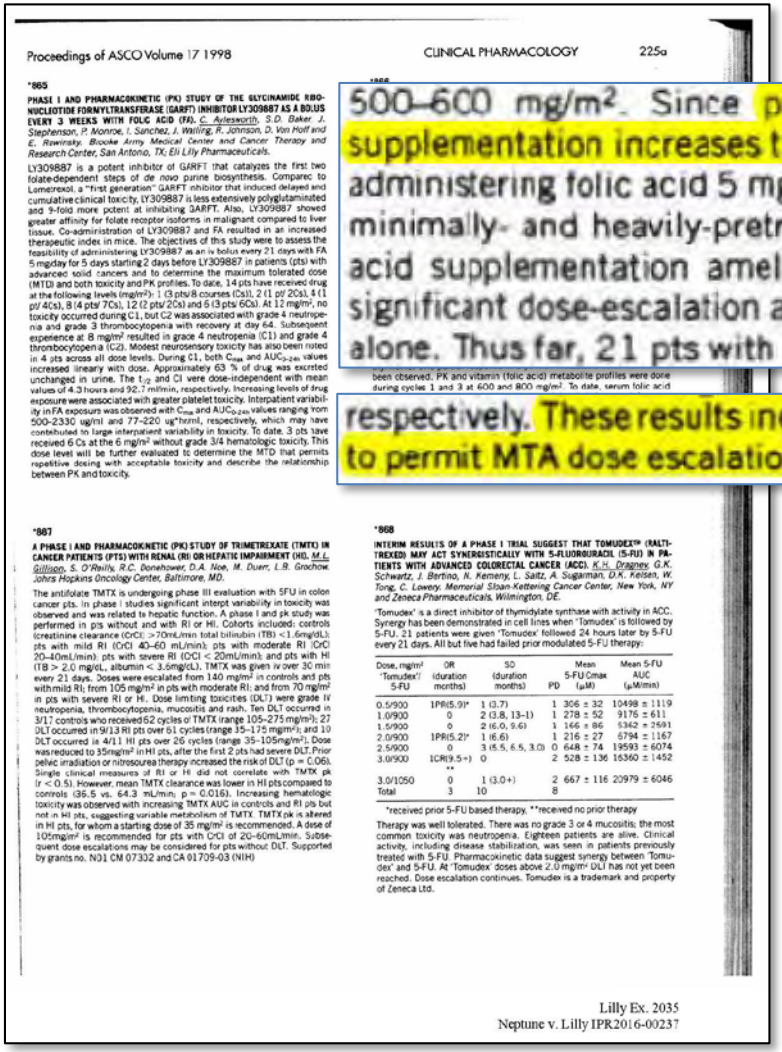
NEPTUNE GENERICS 1022 - 00005

FA, MTA and the Therapeutic Index

Hammond II



Proceedings of ASCO Volume 17 1998 CLINICAL PHARMACOLOGY 225a

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PHASE I AND PHARMACOKINETIC (PK) STUDY OF THE GLYCINAMIDE RIBONUCLEOTIDE FORMYLTRANSFERASE (GARFT) INHIBITOR LY309887 AS A BOLUS EVERY 3 WEEKS WITH FOLIC ACID (FA). C. Aylesworth, S.D. Baker, J. Stephenson, P. Monroe, I. Sanchez, J. Walling, R. Johnson, D. Von Hoff and E. Rowinsky, Brooke Army Medical Center and Cancer Therapy and Research Center, San Antonio, TX; Eli Lilly Pharmaceuticals.

LY309887 is a potent inhibitor of GARFT that catalyzes the first two folate-dependent steps of de novo purine biosynthesis. Compared to Lometrexol, a "first generation" GARFT inhibitor that induced delayed and cumulative clinical toxicity, LY309887 is less extensively polyglutaminated and 9-fold more potent at inhibiting GARFT. Also, LY309887 showed greater affinity for folate receptor isoforms in malignant compared to liver tissue. Co-administration of LY309887 and FA resulted in an increased therapeutic index in mice. The objectives of this study were to assess the feasibility of administering LY309887 as an iv bolus every 21 days with FA 5 mg/day for 5 days starting 2 days before LY309887 in patients (pts) with advanced solid cancers and to determine the maximum tolerated dose (MTD) and both toxicity and PK profiles. To date, 14 pts have received drug at the following levels (mg/m²): 1 (3 pts/ 8 courses (Cs)), 2 (1 pt/ 2Cs), 4 (1 pt/ 4Cs), 8 (4 pts/ 7Cs), 12 (2 pts/ 2Cs) and 6 (3 pts/ 6Cs). At 12 mg/m², no toxicity occurred during C1, but C2 was associated with grade 4 neutropenia and grade 3 thrombocytopenia with recovery at day 64. Subsequent experience at 8 mg/m² resulted in grace 4 neutropenia (C1) and grade 4 thrombocytopenia (C2). Modest neurosensory toxicity has also been noted in 4 pts across all dose levels. During C1, both C_{max} and AUC_{0-24h} values increased linearly with dose. Approximately 63 % of drug was excreted unchanged in urine. The $t_{1/2}$ and Cl were dose-independent with mean values of 4.3 hours and 92.7 ml/min, respectively. Increasing levels of drug exposure were associated with greater platelet toxicity. Interpatient variability in FA exposure was observed with C_{ma} and AUC_{0-24h} values ranging from 500-2330 ug/ml and 77-220 ug*hr/ml, respectively, which may have contributed to large interpatient variability in toxicity. To date, 3 pts have received 6 Cs at the 6 mg/m² without grade 3/4 hematologic toxicity. This dose level will be further evaluated to determine the MTD that permits repetitive dosing with acceptable toxicity and describe the relationship between PK and toxicity.

*867
A PHASE I AND PHARMACOKINETIC (PK) STUDY OF TRIMETREXATE (TMTX) IN CANCER PATIENTS (PTS) WITH RENAL (RI) OR HEPATIC (HI) IMPAIRMENT (HD). M.L. Gilliam, S. O'Reilly, R.C. Donehower, D.A. Noe, M. Duerr, L.B. Grochow, Johns Hopkins Oncology Center, Baltimore, MD.

The antifolate TMTX is undergoing phase III evaluation with 5FU in colon cancer pts. In phase I studies significant interpt variability in toxicity was observed and was related to hepatic function. A phase I and pk study was performed in pts without and with RI or HI. Cohorts included: controls (creatinine clearance (CrCl): >70mL/min total bilirubin (TB) <1.6mg/dL); pts with mild RI (CrCl 40–60 mL/min); pts with moderate RI (CrCl 20–40mL/min); pts with severe RI (CrCl < 20mL/min); and pts with HI (TB > 2.0 mg/dL, albumin < 3.5mg/dL). TMTX was given iv over 30 min every 21 days. Doses were escalated from 140 mg/m² in controls and pts with mild RI; from 105 mg/m² in pts with moderate RI; and from 70 mg/m² in pts with severe RI or HI. Dose limiting toxicities (DLT) were grade IV neutropenia, thrombocytopenia, mucositis and rash. Ten DLT occurred in 3/17 controls who received 62 cycles of TMTX (range 105–275 mg/m²); 27 DLT occurred in 9/13 RI pts over 61 cycles (range 35–175 mg/m²); and 10 DLT occurred is 4/11 HI pts over 26 cycles (range 35–105mg/m²). Dose was reduced to 35mg/m² in HI pts, after the first 2 pts had severe DLT. Prior pelvic irradiation or nitrosourea therapy increased the risk of DLT ($p = 0.06$). Single clinical measures of RI or HI did not correlate with TMTX pk ($r < 0.5$). However, mean TMTX clearance was lower in HI pts compared to controls (36.5 vs. 64.3 mL/min; $p = 0.016$). Increasing hematologic toxicity was observed with increasing TMTX AUC in controls and RI pts but not in HI pts, suggesting variable metabolism of TMTX. TMTX pk is altered in HI pts, for whom a starting dose of 35 mg/m² is recommended. A dose of 140mg/m² is recommended for pts with CrCl of 20–60mL/min. Subsequent dose escalations may be considered for pts without DLT. Supported by grants no. N01 CM 07302 and CA 01709-03 (NIH)

*868
INTERIM RESULTS OF A PHASE I TRIAL SUGGEST THAT TOMUDEX® (RALTITREXED) MAY ACT SYNERGISTICALLY WITH 5-FLUOROURACIL (5-FU) IN PATIENTS WITH ADVANCED COLORECTAL CANCER (ACC). K.H. Dragnev, G.K. Schwartz, J. Bertino, N. Kemeny, L. Saltz, A. Sugarman, D.K. Kelsen, W. Tong, C. Lowery, Memorial Sloan-Kettering Cancer Center, New York, NY and Zeneca Pharmaceuticals, Wilmington, DE.

'Tomudex' is a direct inhibitor of thymidylate synthase with activity in ACC. Synergy has been demonstrated in cell lines when 'Tomudex' is followed by 5-FU. 21 patients were given 'Tomudex' followed 24 hours later by 5-FU every 21 days. All but five had failed prior modulated 5-FU therapy:

Dose, mg/m² 'Tomudex'/ 5-FU	OR (duration months)	SD (duration months)	PD	Mean 5-FU Cmax (μM)	Mean 5-FU AUC (μM/min)
0.5/900	1PR(5.9)*	1 (3.7)	1	306 ± 32	10498 ± 1119
1.0/900	0	2 (3.8, 13-1)	1	278 ± 52	9176 ± 611
1.5/900	0	2 (6.0, 9.6)	1	166 ± 86	5362 ± 2691
2.0/900	1PR(5.2)*	1 (6.6)	1	216 ± 27	6794 ± 1167
2.5/900	0	3 (5.5, 6.5, 3.0)	0	648 ± 74	19593 ± 6074
3.0/900	1CR(9.5+) **	0	2	528 ± 136	16360 ± 1452
3.0/1050	0	1 (3.0+)	2	667 ± 116	20979 ± 6046
Total	3	10	8		

*received prior 5-FU based therapy, **received no prior therapy

Therapy was well tolerated. There was no grade 3 or 4 mucositis; the most common toxicity was neutropenia. Eighteen patients are alive. Clinical activity, including disease stabilization, was seen in patients previously treated with 5-FU. Pharmacokinetic data suggest synergy between 'Tomudex' and 5-FU. At 'Tomudex' doses above 2.0 mg/m² DLT has not yet been reached. Dose escalation continues. Tomudex is a trademark and property of Zeneca Ltd.

Lilly Ex. 2035
Neptune v. Lilly IPR2016-00237

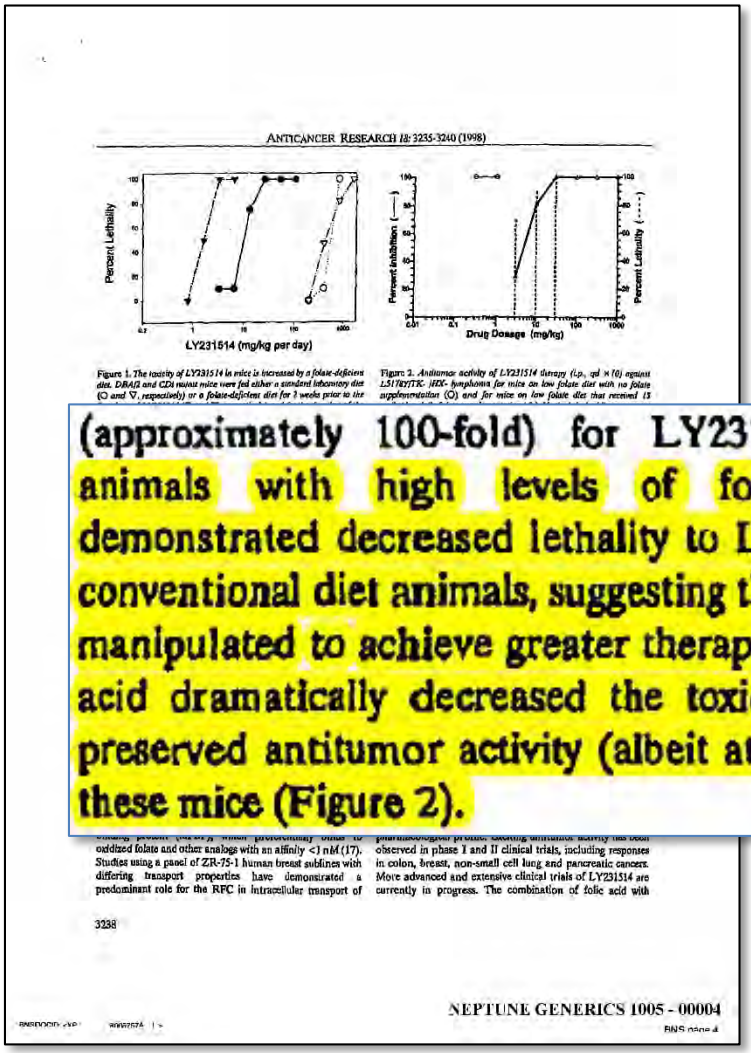
500–600 mg/m². Since preclinical studies indicated that folic acid supplementation increases the therapeutic index of MTA, the feasibility of administering folic acid 5 mg daily for 5 days starting 2 days before MTA in minimally- and heavily-pretreated pts was evaluated to determine if folic acid supplementation ameliorates the toxic 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

been observed. PK and vitamin (folic acid) metabolite profiles were done during cycles 1 and 3 at 600 and 800 mg/m². To date, serum folic acid

respectively. These results indicate that folic acid supplementation appears to permit MTA dose escalation.

FA, MTA and the Therapeutic Index

Worzalla



ANTICANCER RESEARCH *18*: 3235-3240 (1998)

Figure 1. *The toxicity of LY231514 in mice is increased by a folate-deficient diet. DBA/2 and CD1 nu/nu mice were fed either a standard laboratory diet (○ and ▽, respectively) or a folate-deficient diet for 3 weeks prior to the*

Figure 2. *Antitumor activity of LY231514 therapy (i.p., qd × 10) against L5178Y/TK- /HX- lymphoma for mice on low folate diet with no folate supplementation (○) and for mice on low folate diet that received 15*

(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).

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

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

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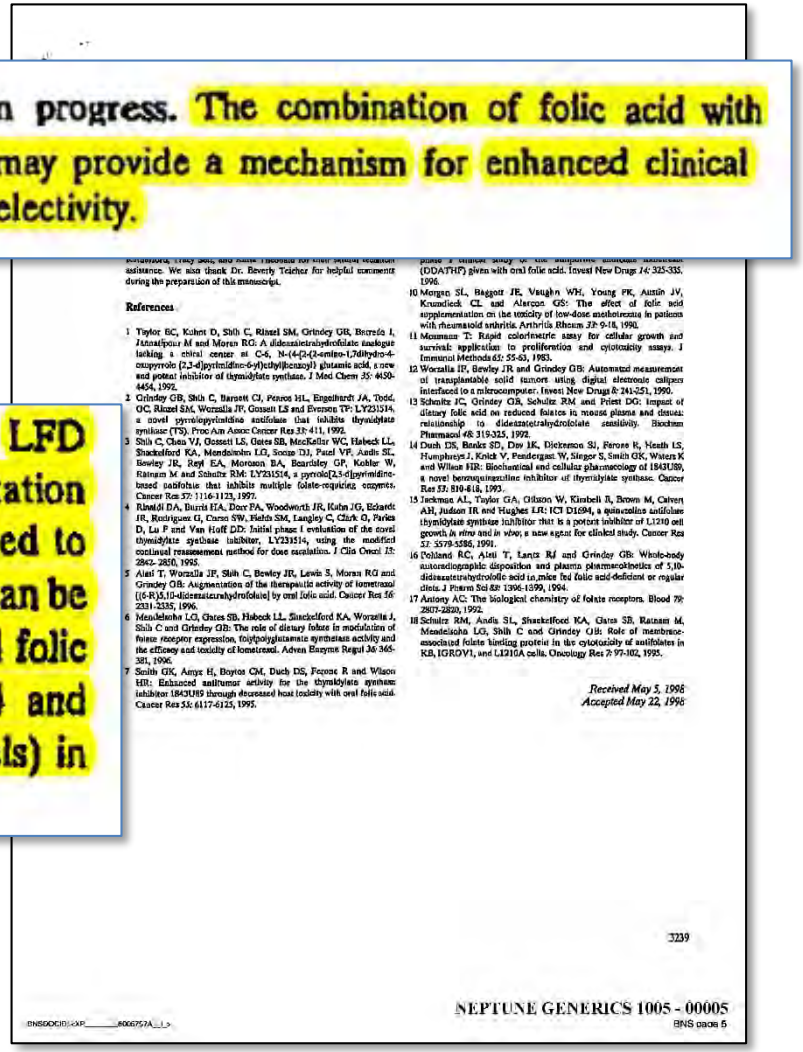
currently in progress. The combination of folic acid with LY231514 may provide a mechanism for enhanced clinical antitumor selectivity.



assistance. We also thank Dr. Beverly Teicher for helpful comments during the preparation of this manuscript.

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3239

NEPTUNE GENERICS 1005 - 00005

B12 + FA

B12 + Folic Acid

EP 005

Furthermore, applicant has surprisingly found that for purposes of controlling blood homocysteine levels, the combination in accordance with the invention of PL, folate and vitamin B12 produces advantageous effects which go substantially beyond what might be expected from a simple additive effect of the action of these drugs. Thus, an unexpected synergism exists when vitamin B12, folate and PL are given concurrently and this effect can be even greater when the vitamins are given in conjunction with a biological

The composition according to the invention is nearly twice as effective as folate alone. This indicates a significantly more than a purely additive effect of the three component combination (synergism).

The tests show that (in contrast to prior art reports teaching the use of folate alone at levels 5 to 20 times higher than in the present trials), nearly 50% of patients do not respond sufficiently to folate alone and 10-20% do not respond to folate alone at all, (not even if the folate dosage rate is greatly increased). By way of contrast, the combination in accordance with the invention, using very low folate concentrations, achieved close on 100% success.

Brattström

Hyperhomocysteinemia due to vitamin B-12 deficiency does not respond to folic acid therapy (Allen et al. 1990). It is likely, that even in subjects with low normal vitamin B-12 concentrations full response to folic acid cannot be achieved unless vitamin B-12 is given concomitantly (Landgren et al. 1995). This view is supported by recent studies by Ubbink et al. (1993a, 1993b, 1994). It was shown that men with moderate

lowering? For several reasons, it seems wise to combine folic acid and cyanocobalamin. First, folic acid seems to reduce almost all but low homocysteine levels. Second, cyanocobalamin will probably secure full folic acid responsiveness. Third, in vitamin B-12 deficiency, erro-

Bronstrup I

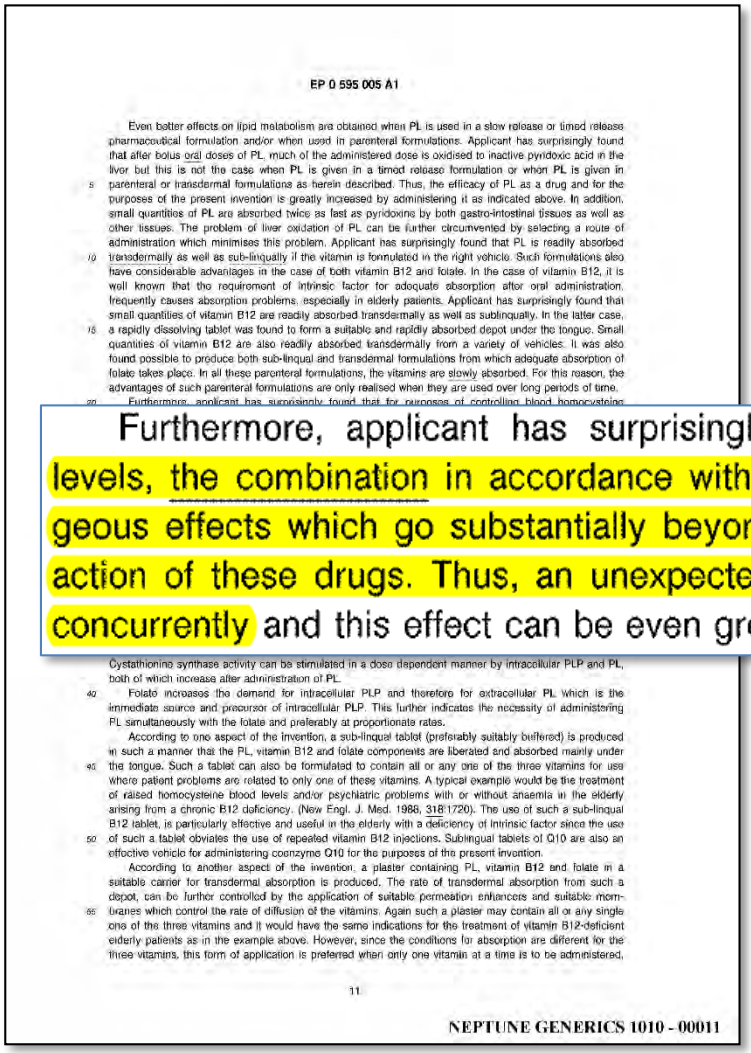
In this study, vitamin B-12 supplementation increased the tHcy-lowering potential of folic acid; this was especially obvious

nmol/L. Because folate and vitamin B-12 have a synergistic function as cofactors of methionine synthase, sufficiency of both seems to be important to increase enzyme activity, whereas a higher availability of only one cofactor, especially in subjects with an already good supply of this cofactor, might lead to only a limited increase in enzyme activity.

Source: Ex. 1010 at 11:20-24, 18:25-26, 40-44; Ex. 1063 at 1277S-78S; Ex. 1019 at 1109; Paper 1 at 19; Paper 48 at 21.

B12 + Folic Acid

EP 005



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Even better effects on lipid metabolism are obtained when PL is used in a slow release or timed release pharmaceutical formulation and/or when used in parenteral formulations. Applicant has surprisingly found that after bolus oral doses of PL much of the administered dose is oxidised to inactive pyridoxic acid in the liver but this is not the case when PL is given in a timed release formulation or when PL is given in parenteral or transdermal formulations as herein described. Thus, the efficacy of PL as a drug and for the purposes of the present invention is greatly increased by administering it as indicated above. In addition, small quantities of PL are absorbed twice as fast as pyridoxine by both gastro-intestinal tissues as well as other tissues. The problem of liver oxidation of PL can be further circumvented by selecting a route of administration which minimises this problem. Applicant has surprisingly found that PL is readily absorbed transdermally as well as sub-lingually if the vitamin is formulated in the right vehicle. Such formulations also have considerable advantages in the case of both vitamin B12 and folate. In the case of vitamin B12, it is well known that the requirement of intrinsic factor for adequate absorption after oral administration frequently causes absorption problems, especially in elderly patients. Applicant has surprisingly found that small quantities of vitamin B12 are readily absorbed transdermally as well as sublingually. In the latter case, a rapidly dissolving tablet was found to form a suitable and rapidly absorbed depot under the tongue. Small quantities of vitamin B12 are also readily absorbed transdermally from a variety of vehicles. It was also found possible to produce both sub-linqual and transdermal formulations from which adequate absorption of folate takes place. In all these parenteral formulations, the vitamins are slowly absorbed. For this reason, the advantages of such parenteral formulations are only realised when they are used over long periods of time.

Furthermore, applicant has surprisingly found that for purposes of controlling blood homocysteine levels, the combination in accordance with the invention of PL, folate and vitamin B12 produces advantageous effects which go substantially beyond what might be expected from a simple additive effect of the action of these drugs. Thus, an unexpected synergism exists when vitamin B12, folate and PL are given concurrently and this effect can be even greater when the vitamins are given in conjunction with a biological

Cystathionine synthase activity can be stimulated in a dose dependent manner by intracellular PLP and PL, both of which increase after administration of PL.

Folate increases the demand for intracellular PLP and therefore for extracellular PL which is the immediate source and precursor of intracellular PLP. This further indicates the necessity of administering PL simultaneously with the folate and preferably at proportionate rates.

According to one aspect of the invention, a sub-linqual tablet (preferably suitably buffered) is produced in such a manner that the PL, vitamin B12 and folate components are liberated and absorbed mainly under the tongue. Such a tablet can also be formulated to contain all or any one of the three vitamins for use where patient problems are related to only one of these vitamins. A typical example would be the treatment of raised homocysteine blood levels and/or psychiatric problems with or without anaemia in the elderly arising from a chronic B12 deficiency. (New Engl. J. Med. 1988, 318:1720). The use of such a sub-linqual B12 tablet, is particularly effective and useful in the elderly with a deficiency of intrinsic factor since the use of such a tablet obviates the use of repeated vitamin B12 injections. Sublingual tablets of Q10 are also an effective vehicle for administering coenzyme Q10 for the purposes of the present invention.

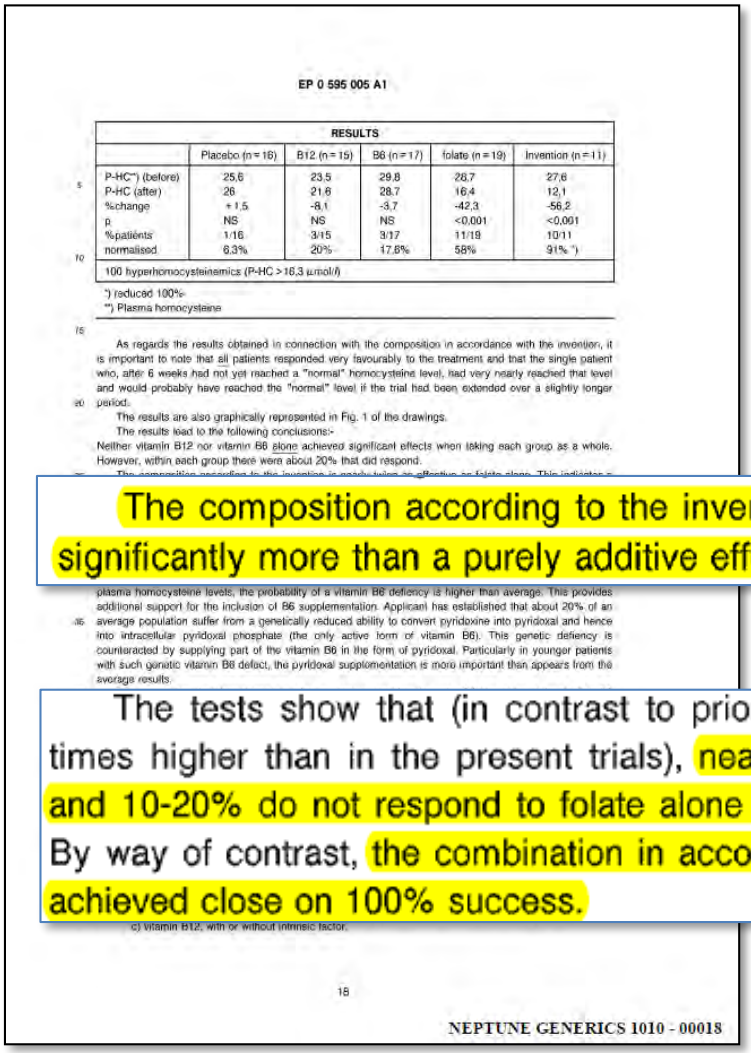
According to another aspect of the invention, a plaster containing PL, vitamin B12 and folate in a suitable carrier for transdermal absorption is produced. The rate of transdermal absorption from such a depot, can be further controlled by the application of suitable permeation enhancers and suitable membranes which control the rate of diffusion of the vitamins. Again such a plaster may contain all or any single one of the three vitamins and it would have the same indications for the treatment of vitamin B12-deficient elderly patients as in the example above. However, since the conditions for absorption are different for the three vitamins, this form of application is preferred when only one vitamin at a time is to be administered.

11

NEPTUNE GENERICS 1010 - 00011

B12 + Folic Acid

EP 005



EP 0 595 005 A1

RESULTS

	Placebo (n = 16)	B12 (n = 15)	B6 (n = 17)	folate (n = 19)	invention (n = 11)
P-HC*) (before)	25,6	23,5	29,8	28,7	27,6
P-HC (after)	26	21,6	28,7	16,4	12,1
%change	+ 1,5	-8,1	-3,7	-42,3	-56,2
p	NS	NS	NS	<0,001	<0,001
%patients normalised	1/16 6,3%	3/15 20%	3/17 17,6%	11/19 58%	10/11 91% **)
100 hyperhomocysteinemics (P-HC > 16,3 µmol/l)					

*) reduced 100%
**) Plasma homocysteine

As regards the results obtained in connection with the composition in accordance with the invention, it is important to note that all patients responded very favourably to the treatment and that the single patient who, after 6 weeks had not yet reached a "normal" homocysteine level, had very nearly reached that level and would probably have reached the "normal" level if the trial had been extended over a slightly longer period.

The results are also graphically represented in Fig. 1 of the drawings.

The results lead to the following conclusions:-

Neither vitamin B12 nor vitamin B6 alone achieved significant effects when taking each group as a whole. However, within each group there were about 20% that did respond.

plasma homocysteine levels, the probability of a vitamin B6 deficiency is higher than average. This provides additional support for the inclusion of B6 supplementation. Applicant has established that about 20% of an average population suffer from a genetically reduced ability to convert pyridoxine into pyridoxal and hence into intracellular pyridoxal phosphate (the only active form of vitamin B6). This genetic deficiency is counteracted by supplying part of the vitamin B6 in the form of pyridoxal. Particularly in younger patients with such genetic vitamin B6 defect, the pyridoxal supplementation is more important than appears from the average results.

c) vitamin B12, with or without intrinsic factor.

18

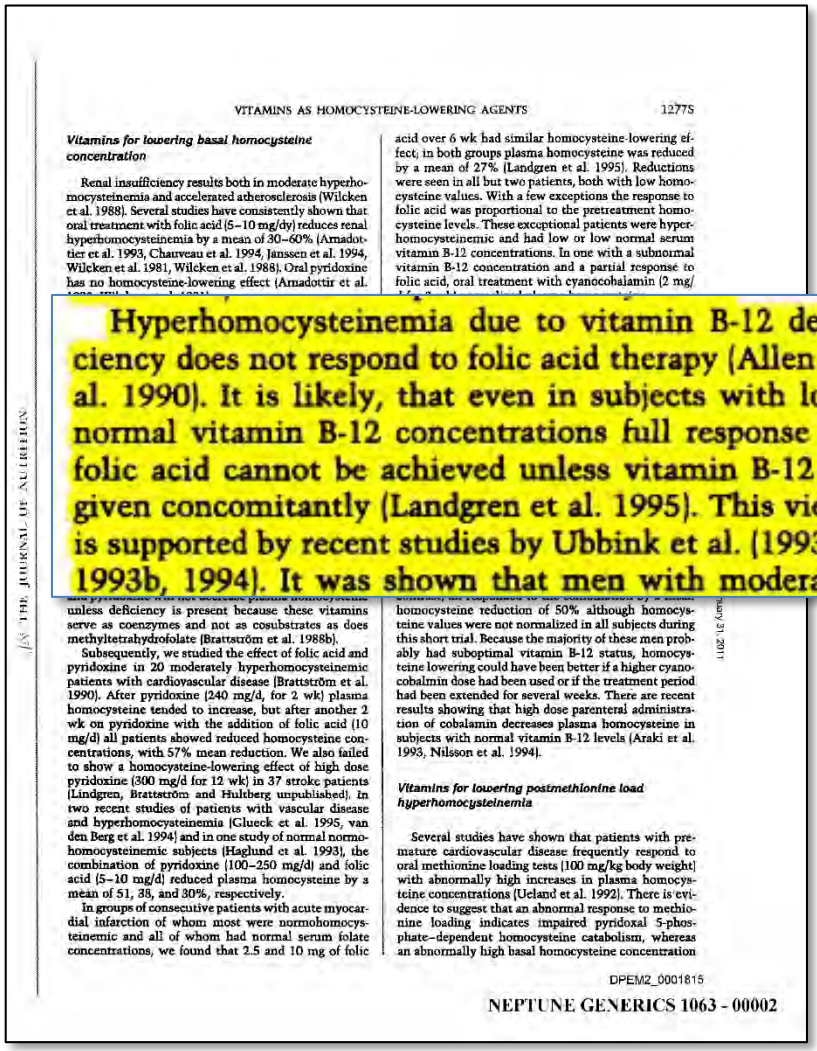
NEPTUNE GENERICS 1010 - 00018

The composition according to the invention is nearly twice as effective as folate alone. This indicates a significantly more than a purely additive effect of the three component combination (synergism).

The tests show that (in contrast to prior art reports teaching the use of folate alone at levels 5 to 20 times higher than in the present trials), nearly 50% of patients do not respond sufficiently to folate alone and 10-20% do not respond to folate alone at all, (not even if the folate dosage rate is greatly increased). By way of contrast, the combination in accordance with the invention, using very low folate concentrations, achieved close on 100% success.

B12 + Folic Acid

Brattström



VITAMINS AS HOMOCYSTEINE-LOWERING AGENTS 1277S

Vitamins for lowering basal homocysteine concentration

Renal insufficiency results both in moderate hyperhomocysteinemia and accelerated atherosclerosis (Wilcken et al. 1988). Several studies have consistently shown that oral treatment with folic acid (5–10 mg/dy) reduces renal hyperhomocysteinemia by a mean of 30–60% (Arnadottier et al. 1993, Chauveau et al. 1994, Janssen et al. 1994, Wilcken et al. 1981, Wilcken et al. 1988). Oral pyridoxine has no homocysteine-lowering effect (Arnadottir et al.

Hyperhomocysteinemia due to vitamin B-12 deficiency does not respond to folic acid therapy (Allen et al. 1990). It is likely, that even in subjects with low normal vitamin B-12 concentrations full response to folic acid cannot be achieved unless vitamin B-12 is given concomitantly (Landgren et al. 1995). This view is supported by recent studies by Ubbink et al. (1993a, 1993b, 1994). It was shown that men with moderate

unless deficiency is present because these vitamins serve as coenzymes and not as cosubstrates as does methyltetrahydrofolate (Brattström et al. 1988b).

Subsequently, we studied the effect of folic acid and pyridoxine in 20 moderately hyperhomocysteinemic patients with cardiovascular disease (Brattström et al. 1990). After pyridoxine (240 mg/d, for 2 wk) plasma homocysteine tended to increase, but after another 2 wk on pyridoxine with the addition of folic acid (10 mg/d) all patients showed reduced homocysteine concentrations, with 57% mean reduction. We also failed to show a homocysteine-lowering effect of high dose pyridoxine (300 mg/d for 12 wk) in 37 stroke patients (Lindgren, Brattström and Hultberg unpublished). In two recent studies of patients with vascular disease and hyperhomocysteinemia (Glueck et al. 1995, van den Berg et al. 1994) and in one study of normal normohomocysteinemic subjects (Haglund et al. 1993), the combination of pyridoxine (100–250 mg/d) and folic acid (5–10 mg/d) reduced plasma homocysteine by a mean of 51, 38, and 30%, respectively.

In groups of consecutive patients with acute myocardial infarction of whom most were normohomocysteinemic and all of whom had normal serum folate concentrations, we found that 2.5 and 10 mg of folic acid over 6 wk had similar homocysteine-lowering effect; in both groups plasma homocysteine was reduced by a mean of 27% (Landgren et al. 1995). Reductions were seen in all but two patients, both with low homocysteine values. With a few exceptions the response to folic acid was proportional to the pretreatment homocysteine levels. These exceptional patients were hyperhomocysteinemic and had low or low normal serum vitamin B-12 concentrations. In one with a subnormal vitamin B-12 concentration and a partial response to folic acid, oral treatment with cyanocobalamin (2 mg/

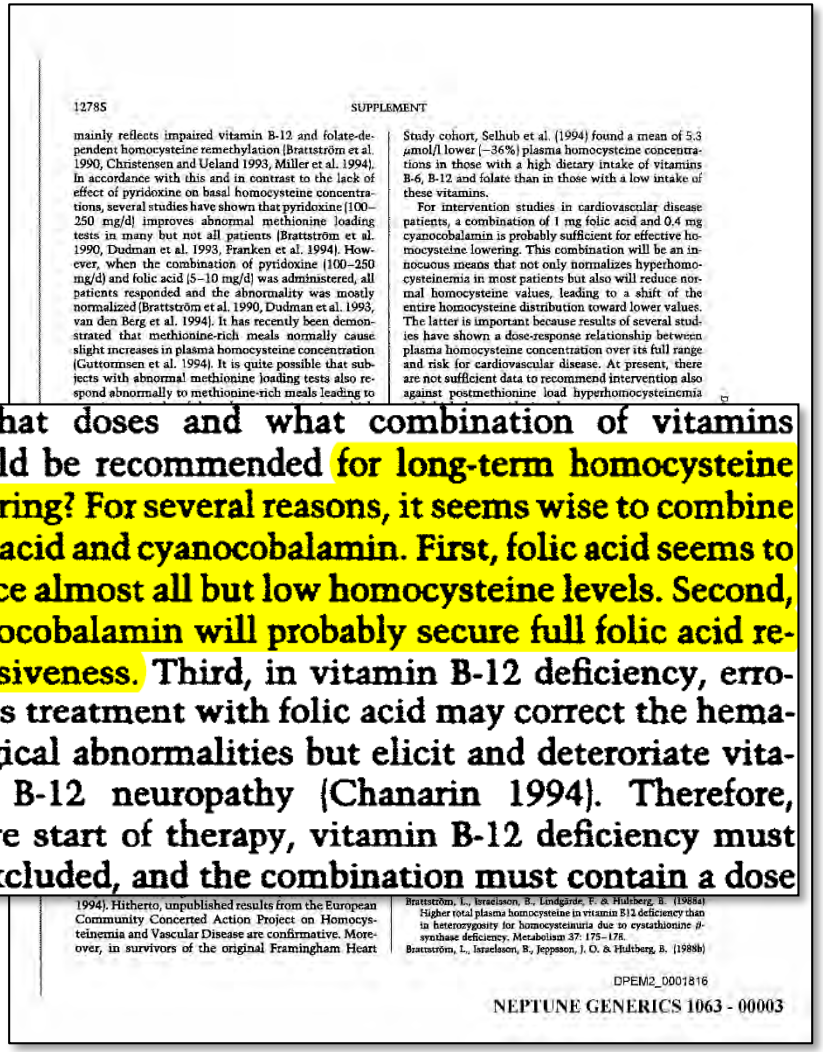
homocysteine reduction of 50% although homocysteine values were not normalized in all subjects during this short trial. Because the majority of these men probably had suboptimal vitamin B-12 status, homocysteine lowering could have been better if a higher cyanocobalmin dose had been used or if the treatment period had been extended for several weeks. There are recent results showing that high dose parenteral administration of cobalamin decreases plasma homocysteine in subjects with normal vitamin B-12 levels (Araki et al. 1993, Nilsson et al. 1994).

Vitamins for lowering postmethionine load hyperhomocysteinemia

Several studies have shown that patients with premature cardiovascular disease frequently respond to oral methionine loading tests (100 mg/kg body weight) with abnormally high increases in plasma homocysteine concentrations (Ueland et al. 1992). There is evidence to suggest that an abnormal response to methionine loading indicates impaired pyridoxal 5-phosphate–dependent homocysteine catabolism, whereas an abnormally high basal homocysteine concentration

DPEM2_0001815

NEPTUNE GENERICS 1063 - 00002



1278S SUPPLEMENT

mainly reflects impaired vitamin B-12 and folate-dependent homocysteine remethylation (Brattström et al. 1990, Christensen and Ueland 1993, Miller et al. 1994). In accordance with this and in contrast to the lack of effect of pyridoxine on basal homocysteine concentrations, several studies have shown that pyridoxine (100–250 mg/d) improves abnormal methionine loading tests in many but not all patients (Brattström et al. 1990, Dudman et al. 1993, Franken et al. 1994). However, when the combination of pyridoxine (100–250 mg/d) and folic acid (5–10 mg/d) was administered, all patients responded and the abnormality was mostly normalized (Brattström et al. 1990, Dudman et al. 1993, van den Berg et al. 1994). It has recently been demonstrated that methionine-rich meals normally cause slight increases in plasma homocysteine concentration (Guttormsen et al. 1994). It is quite possible that subjects with abnormal methionine loading tests also respond abnormally to methionine-rich meals leading to

Study cohort, Selhub et al. (1994) found a mean of 5.3 μmol/l lower (−36%) plasma homocysteine concentrations in those with a high dietary intake of vitamins B-6, B-12 and folate than in those with a low intake of these vitamins.

For intervention studies in cardiovascular disease patients, a combination of 1 mg folic acid and 0.4 mg cyanocobalamin is probably sufficient for effective homocysteine lowering. This combination will be an innocuous means that not only normalizes hyperhomocysteinemia in most patients but also will reduce normal homocysteine values, leading to a shift of the entire homocysteine distribution toward lower values. The latter is important because results of several studies have shown a dose-response relationship between plasma homocysteine concentration over its full range and risk for cardiovascular disease. At present, there are not sufficient data to recommend intervention also against postmethionine load hyperhomocysteinemia

What doses and what combination of vitamins should be recommended for long-term homocysteine lowering? For several reasons, it seems wise to combine folic acid and cyanocobalamin. First, folic acid seems to reduce almost all but low homocysteine levels. Second, cyanocobalamin will probably secure full folic acid responsiveness. Third, in vitamin B-12 deficiency, erroneous treatment with folic acid may correct the hematological abnormalities but elicit and deteroriate vitamin B-12 neuropathy (Chanarin 1994). Therefore, before start of therapy, vitamin B-12 deficiency must be excluded, and the combination must contain a dose

1994). Hitherto, unpublished results from the European Community Concerted Action Project on Homocysteinemia and Vascular Disease are confirmative. Moreover, in survivors of the original Framingham Heart

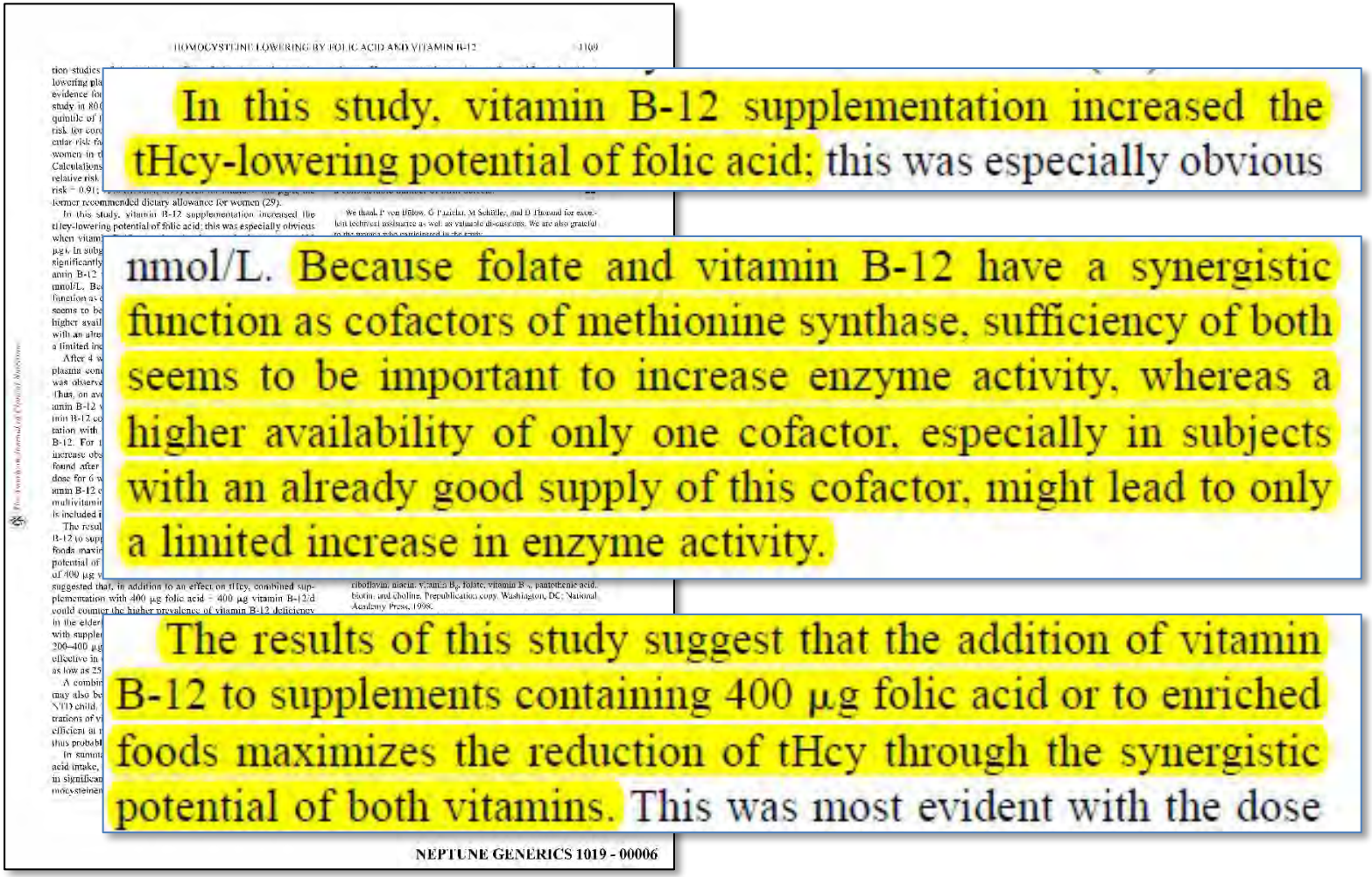
Brattström, L., Israelsson, B., Lindgärde, F. & Hultberg, B. (1988a) Higher total plasma homocysteine in vitamin B12 deficiency than in heterozygosity for homocysteinuria due to cystathionine β-synthase deficiency. Metabolism 37: 175–178.
Brattström, L., Israelsson, B., Jeppsson, J. O. & Hultberg, B. (1988b)

DPEM2_0001816

NEPTUNE GENERICS 1063 - 00003

B12 + Folic Acid

Bronstrup I



HOMOCYSTEINE LOWERING BY FOLIC ACID AND VITAMIN B-12 1109

In this study, vitamin B-12 supplementation increased the tHcy-lowering potential of folic acid; this was especially obvious

nmol/L. Because folate and vitamin B-12 have a synergistic function as cofactors of methionine synthase, sufficiency of both seems to be important to increase enzyme activity, whereas a higher availability of only one cofactor, especially in subjects with an already good supply of this cofactor, might lead to only a limited increase in enzyme activity.

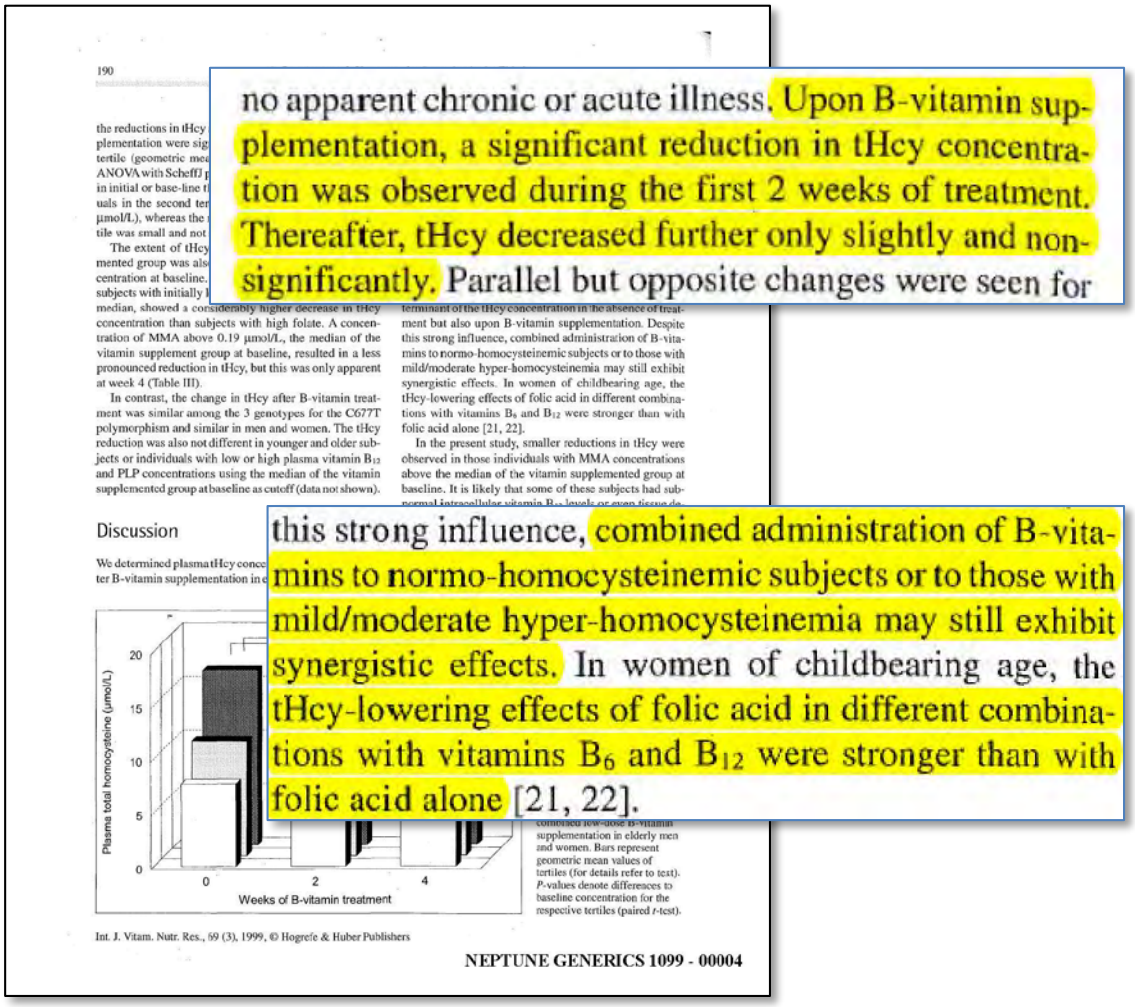
The results of this study suggest that the addition of vitamin B-12 to supplements containing 400 μg folic acid or to enriched foods maximizes the reduction of tHcy through the synergistic potential of both vitamins. This was most evident with the dose

NEPTUNE GENERICS 1019 - 00006

Source: Ex. 1019 at 1109; Paper 48 at 21.

B12 + Folic Acid

Bronstrup II



190

the reductions in tHcy
plementation were sig
tertile (geometric mea
ANOVA with Scheffé p
in initial or base-line t
uals in the second ter
µmol/L), whereas the
tile was small and not

The extent of tHcy
mented group was als
centration at baseline.
subjects with initially
median, showed a considerably higher decrease in tHcy concentration than subjects with high folate. A concentration of MMA above 0.19 µmol/L, the median of the vitamin supplement group at baseline, resulted in a less pronounced reduction in tHcy, but this was only apparent at week 4 (Table III).

In contrast, the change in tHcy after B-vitamin treatment was similar among the 3 genotypes for the C677T polymorphism and similar in men and women. The tHcy reduction was also not different in younger and older subjects or individuals with low or high plasma vitamin B_{12} and PLP concentrations using the median of the vitamin supplemented group at baseline as cutoff (data not shown).

no apparent chronic or acute illness. Upon B-vitamin supplementation, a significant reduction in tHcy concentration was observed during the first 2 weeks of treatment. Thereafter, tHcy decreased further only slightly and non-significantly. Parallel but opposite changes were seen for

terminant of the tHcy concentration in the absence of treatment but also upon B-vitamin supplementation. Despite this strong influence, combined administration of B-vitamins to normo-homocysteinemic subjects or to those with mild/moderate hyper-homocysteinemia may still exhibit synergistic effects. In women of childbearing age, the tHcy-lowering effects of folic acid in different combinations with vitamins B_6 and B_{12} were stronger than with folic acid alone [21, 22].

In the present study, smaller reductions in tHcy were observed in those individuals with MMA concentrations above the median of the vitamin supplemented group at baseline. It is likely that some of these subjects had sub-

Discussion

We determined plasma tHcy conce
ter B-vitamin supplementation in

this strong influence, combined administration of B-vitamins to normo-homocysteinemic subjects or to those with mild/moderate hyper-homocysteinemia may still exhibit synergistic effects. In women of childbearing age, the tHcy-lowering effects of folic acid in different combinations with vitamins B_6 and B_{12} were stronger than with folic acid alone [21, 22].

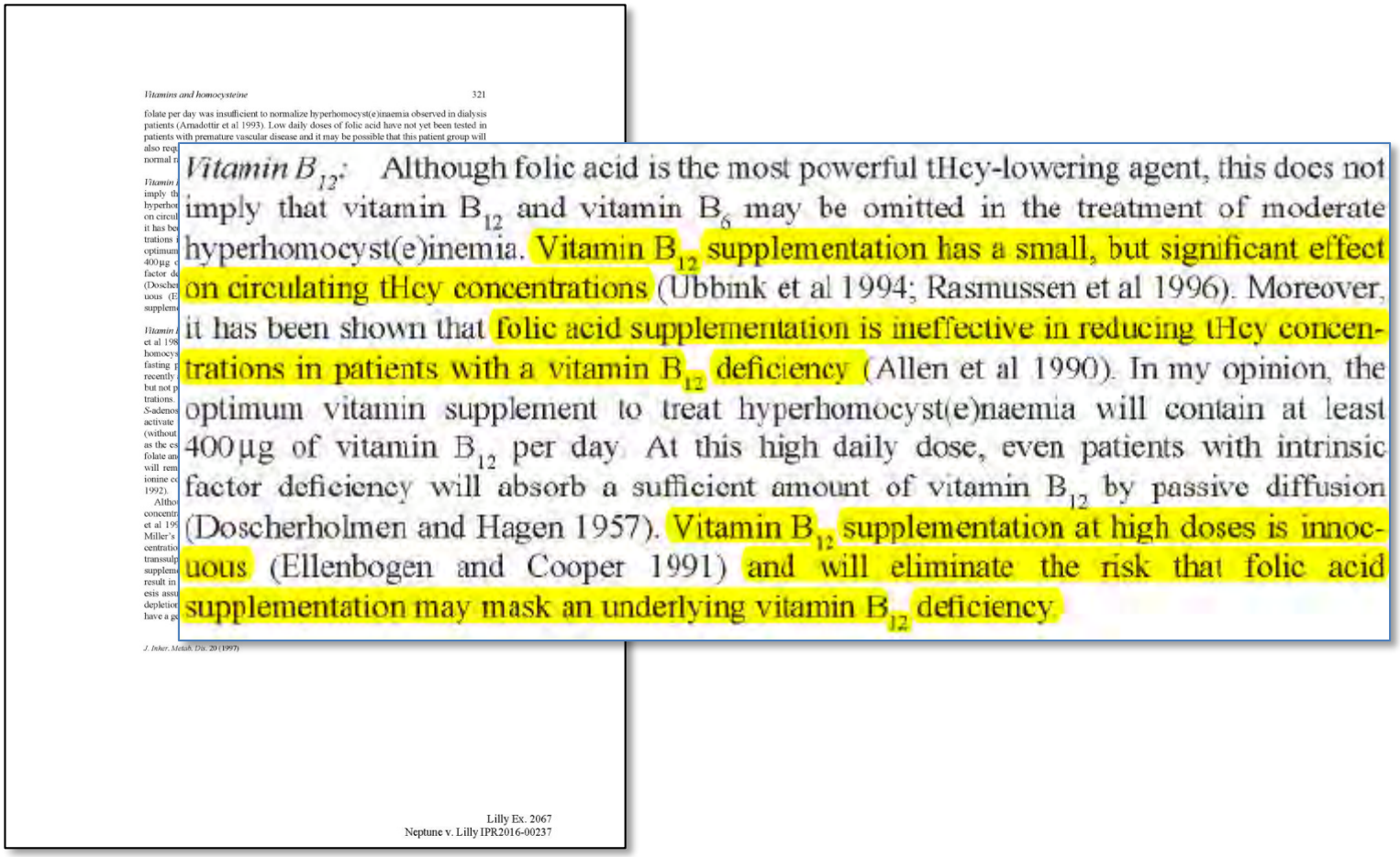
supplementation in elderly men and women. Bars represent geometric mean values of tertiles (for details refer to text). *P*-values denote differences to baseline concentration for the respective tertiles (paired *t*-test).

Int. J. Vitam. Nutr. Res., 69 (3), 1999, © Hogrefe & Huber Publishers

Source: Ex. 1099 at 190; Paper 48 at 21.

B12 + Folic Acid

Ubbink II



Vitamins and homocysteine 321

folate per day was insufficient to normalize hyperhomocyst(e)inaemia observed in dialysis patients (Arnadottir et al 1993). Low daily doses of folic acid have not yet been tested in patients with premature vascular disease and it may be possible that this patient group will

Vitamin B_{12}: Although folic acid is the most powerful tHcy-lowering agent, this does not imply that vitamin B_{12} and vitamin B_6 may be omitted in the treatment of moderate hyperhomocyst(e)inemia. Vitamin B_{12} supplementation has a small, but significant effect on circulating tHcy concentrations (Ubbink et al 1994; Rasmussen et al 1996). Moreover, it has been shown that folic acid supplementation is ineffective in reducing tHcy concentrations in patients with a vitamin B_{12} deficiency (Allen et al 1990). In my opinion, the optimum vitamin supplement to treat hyperhomocyst(e)naemia will contain at least 400 µg of vitamin B_{12} per day. At this high daily dose, even patients with intrinsic factor deficiency will absorb a sufficient amount of vitamin B_{12} by passive diffusion (Doscherholmen and Hagen 1957). Vitamin B_{12} supplementation at high doses is innocuous (Ellenbogen and Cooper 1991) and will eliminate the risk that folic acid supplementation may mask an underlying vitamin B_{12} deficiency.

J. Inher. Metab. Dis. 20 (1997)

Lilly Ex. 2067
Neptune v. Lilly IPR2016-00237

Source: Ex. 2067 at 321; Paper 48 at 25-26.

DOSE AND SCHEDULE

B12 Dose and Schedule

’209 Patent Specification

in humans; Savage D G, Lindenbaum J, Stabler S P, Allen R H, Sensitivity of methylmalonic acid and total homocysteine determination for diagnosing cobalamin and folate deficiency. Am J Med 1994; 96: 239-246.

The term "vitamin B12" refers to vitamin B12 and its pharmaceutical derivatives, such as hydroxocobalamin, cyano-10-chlorocobalamin, aquocobalamin perchlorate, aquo-10-chlorocobalamin perchlorate, azidocobalamin, chlorocobalamin, and cobalamin. Preferably the term refers to vitamin B12, cobalamin, and chlorocobalamin.

The dosage generally will be provided in the form of a vitamin supplement, namely as a tablet administered orally, such as a sustained release formulation, as an aqueous solution added to drinking water, or as an aqueous parenteral formulation. Preferably the methylmalonic acid lowering agent is administered as an intramuscular injection formulation. Such formulations are known in the art and are commercially available.

The skilled artisan will appreciate that the methylmalonic lowering agents are effective over a wide dosage range. For example, when cobalamin is used as the methylmalonic lowering agent, the dosage of cobalamin may fall within the range of about 0.2 µg to about 3000 µg of cobalamin from once daily for a month to once every nine weeks for a year. Preferably, cobalamin will be dosed as an intramuscular injection of about 500 µg to about 1500 µg administered from about every 24 hours to about every 1680 hours. Preferably, it is an intramuscular injection of about 1000 µg administered initially from about 1 to about 3 weeks prior to administration of the antifolate and repeated from about every 24 hours to about every 1680 hours, regardless of when treatment with the antifolate is started and continued until the administration of the antifolate is discontinued. Most preferred is an intramuscular injection of about 1000 µg administered initially from about 1 to about 3 weeks prior to the first administration of the antifolate and repeated every 6 to 12 weeks, preferably about every 9 weeks, and continued until the discontinuation of the antifolate administrations. However, it will be understood that the amount of the methylmalonic acid lowering agent actually administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual agent administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful side effect.

[illegible] folic binding protein binding agent which includes folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, and (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically-available salt or ester thereof. This latter compound is the (6R)-isomer of leucovorin as disclosed in J. Am. Chem. Soc., 74, 4215 (1952). Both of the tetrahydrofolic acid compounds are in the unnatural configuration at the 6-position. They are 10-20 fold more efficient in binding the folate binding protein compared with their respective (6S)-isomer, see Ratnam, et. al., Folate and Antifolate Transport in Mammalian Cells Symposium, Mar. 21-22, 1991, Bethesda, Md. These compounds are usually prepared as a mixture with their natural form (6S) of diastereomers by non-stereoselective reduction from the corresponding dehydro precursors followed by separation through chromatographic or enzymatic techniques. See e.g. PCT Patent Application Publication WO

880844 (also Derwent Abstract 88-368464/51) and Canadian Patent 1093554, See, e.g. Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline (2000). 8 Folate, pp. 196-305.

"Physiologically-available salt" refers to potassium, sodium, lithium, magnesium, or preferably a calcium salt of the FBP binding agent. "Physiologically-available . . . ester" refers to esters which are easily hydrolyzed upon administration to a mammal to provide the corresponding FBP binding agent free acid, such as C_1-C_4 alkyl esters, mixed anhydrides, and the like.

The FBP binding agent to be utilized according to this invention can be in its free acid form, or can be in the form of a physiologically-acceptable salt or ester which is converted to the parent acid in a biological system. The dosage generally will be provided in the form of a vitamin supplement, namely as a tablet administered orally, preferably as a sustained release formulation, as an aqueous solution added to drinking water, an aqueous parenteral formulation, e.g., an intravenous formulation, or the like.

The FBP binding agent is usually administered to the subject mammal prior to treatment with the antifolate. Pretreatment with the suitable amount of FBP binding agent from about 1 to about 24 hours is usually sufficient to substantially bind to and block the folate binding protein prior to administration of the antifolate. Although one single dose of the FBP binding agent, preferably an oral administration of folic acid, should be sufficient to load the folate binding protein, [illegible] periods up to weeks before treatment with the active agent to ensure that the folate binding protein is sufficiently bound in order to maximize the benefit derived from such pretreatment.

In the especially preferred embodiment of this invention, about 0.1 mg to about 30 mg, most preferably about 0.3 mg to about 5 mg, of folic acid is administered orally to a mammal about 1 to 3 weeks post administration of the methylmalonic acid lowering agent and about 1 to about 24 hours prior to the parenteral administration of the amount of an antifolate. However, it will be understood that the amount of the methylmalonic acid lowering agent actually administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual agent administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful side effect.

In general, the term "pharmaceutical" when used as an adjective means substantially non-toxic to living organisms.

Methods

To assess the effect of a methylmalonic acid lowering agent, alone or in combination with folic acid on the antitumor efficacy of an antifolate in a human tumor xenograft model, female nude mice bearing human MX-1 breast carcinoma were treated with ALIMTA alone or along with super-physiologic doses of folic acid or vitamin B12 (cobalamin).

The animals were maintained on sterilized standard lab chow ad libitum and sterilized water ad libitum. The human MX-1 tumor cells (5×10^6) obtained from donor tumors were implanted subcutaneously in a thigh of female nude mice 8- to 10-weeks old. Beginning on day 7 post tumor cell implanta-

NEPTUNE GENERICS 1001 - 00005

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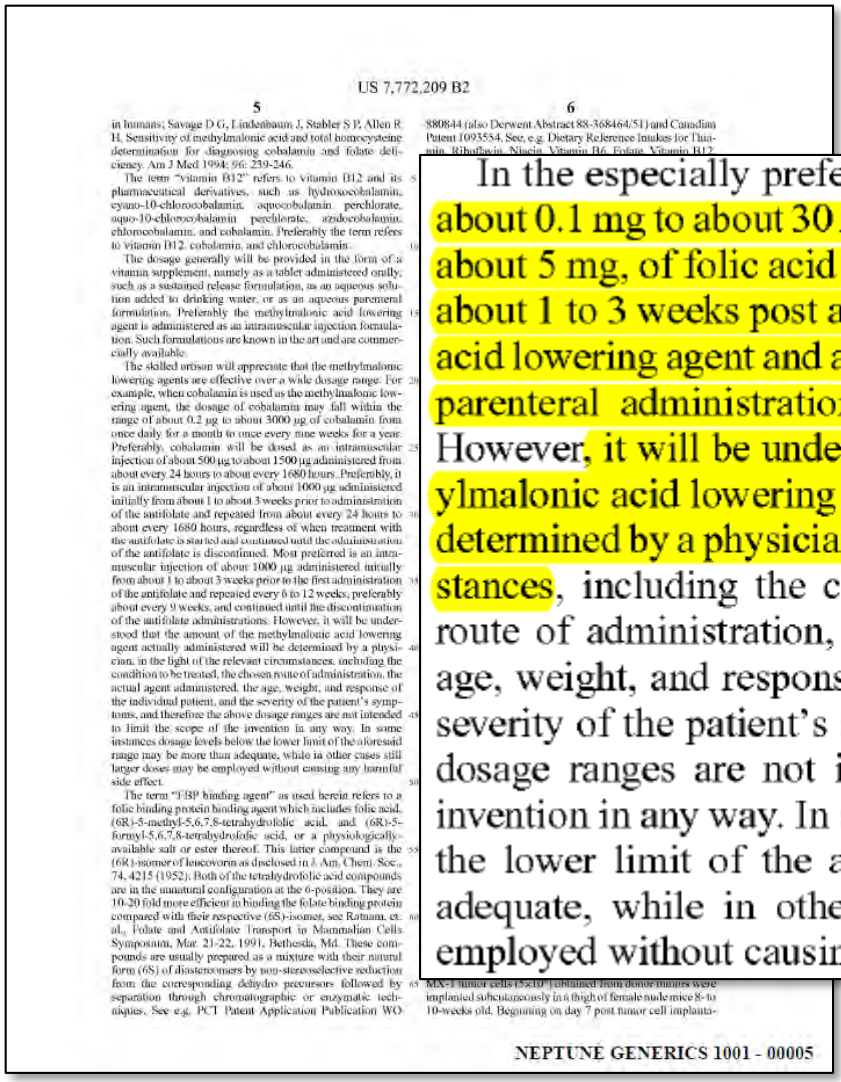
Source: Ex. 1001 at 5:19-50; Paper 1 at 38, 44; Paper 48 at 31.

FA Dose and Schedule

’209 Patent Specification



US 7,772,209 B2

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in humans; Savage D G, Lindenbaum J, Stabler S P, Allen R H. Sensitivity of methylmalonic acid and total homocysteine determination for diagnosing cobalamin and folate deficiency. Am J Med 1994; 96: 239-246.

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The term "FBP binding agent" as used herein refers to a folic binding protein binding agent which includes folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, and (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically-available salt or ester thereof. This latter compound is the (6R)-isomer of leucovorin as disclosed in J. Am. Chem. Soc., 74, 4215 (1952). Both of the tetrahydrofolic acid compounds are in the unnatural configuration at the 6-position. They are 10-20 fold more efficient in binding the folate binding protein compared with their respective (6S)-isomer, see Ratnam, et. al., Folate and Antifolate Transport in Mammalian Cells Symposium, Mar. 21-22, 1991, Bethesda, Md. These compounds are usually prepared as a mixture with their natural form (6S) of diastereomers by non-stereoselective reduction from the corresponding dehydro precursors followed by separation through chromatographic or enzymatic techniques. See e.g. PCT Patent Application Publication WO

6

880844 (also Derwent Abstract 88-368464/51) and Canadian Patent 1093554. See, e.g. Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12,

MX-1 tumor cells ($5x10^6$) obtained from donor tumors were implanted subcutaneously in a thigh of female nude mice 8- to 10-weeks old. Beginning on day 7 post tumor cell implanta-

NEPTUNE GENERICS 1001 - 00005

In the especially preferred embodiment of this invention, about 0.1 mg to about 30 mg, most preferably about 0.3 mg to about 5 mg, of folic acid is administered orally to a mammal about 1 to 3 weeks post administration of the methylmalonic acid lowering agent and about 1 to about 24 hours prior to the parenteral administration of the amount of an antifolate. However, it will be understood that the amount of the methylmalonic acid lowering agent actually administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual agent administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful side effect.

Source: Ex. 1001 at 6:35-52; Paper 1 at 58.

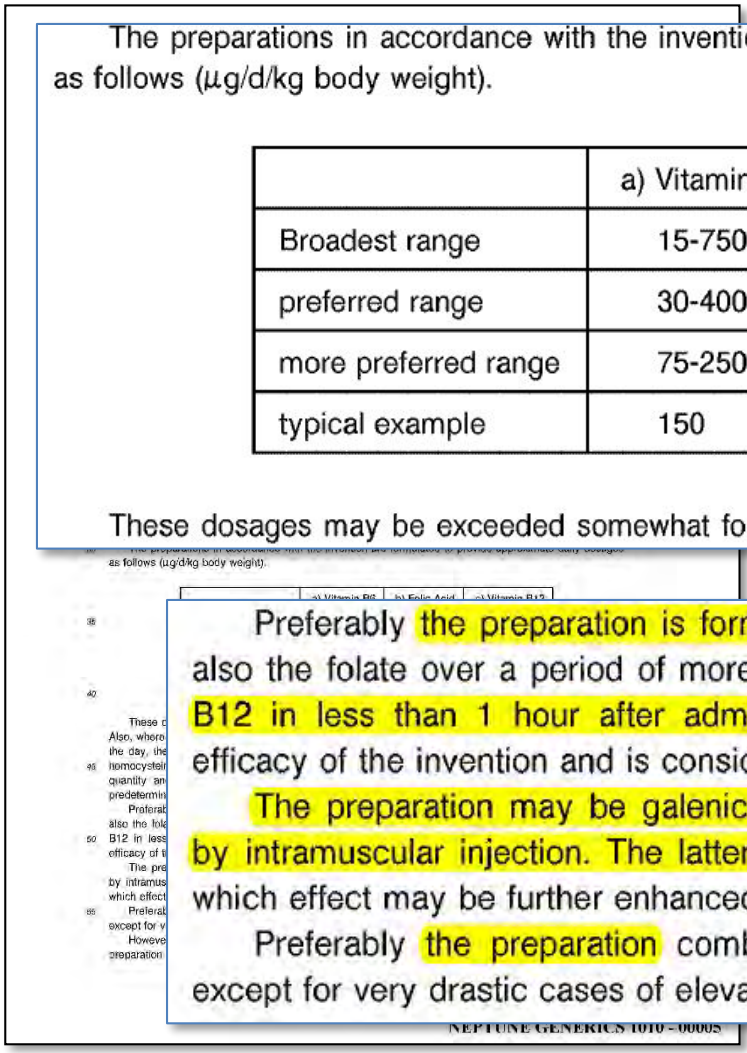
B12 and FA Dosing

EP 005

The preparations in accordance with the invention are formulated to provide approximate daily dosages as follows (µg/d/kg body weight).

	a) Vitamin B6	b) Folic Acid	c) Vitamin B12
Broadest range	15-750	1,5-150	1,5-75
preferred range	30-400	7,5-50	3-15
more preferred range	75-250	10-30	7-10
typical example	150	15	7,5

These dosages may be exceeded somewhat for short durations, e.g. at the beginning of the treatment.



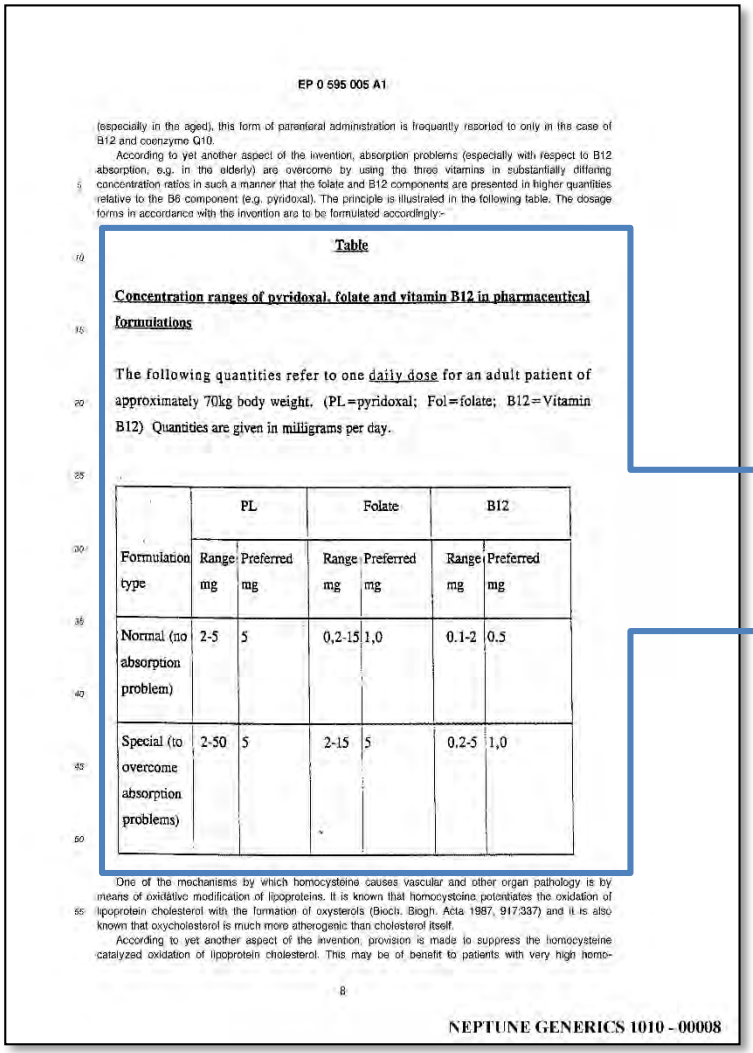
Preferably the preparation is formulated to make available to the patient the vitamin B6 and preferably also the folate over a period of more than 1 hour and to make available an effective dosage of the vitamin B12 in less than 1 hour after administration. This feature is considered to contribute materially to the efficacy of the invention and is considered to be novel and inventive per se.

The preparation may be galenically formulated for parenteral administration, preferably by infusion or by intramuscular injection. The latter form inherently provides for a retarded availability of the ingredients, which effect may be further enhanced by depot forms of formulation.

Preferably the preparation combines all three essential ingredients in a single dosage form, which except for very drastic cases of elevated homocysteine levels is preferably designed for oral administration.

B12 and FA Dosing

EP 005



EP 0 595 005 A1

(especially in the aged), this form of parenteral administration is frequently resorted to only in the case of B12 and coenzyme Q10.

According to yet another aspect of the invention, absorption problems (especially with respect to B12 absorption, e.g. in the elderly) are overcome by using the three vitamins in substantially differing concentration ratios in such a manner that the folate and B12 components are presented in higher quantities relative to the B6 component (e.g. pyridoxal). The principle is illustrated in the following table. The dosage forms in accordance with the invention are to be formulated accordingly:-

Table

Concentration ranges of pyridoxal, folate and vitamin B12 in pharmaceutical formulations

The following quantities refer to one daily dose for an adult patient of approximately 70kg body weight. (PL=pyridoxal; Fol=folate; B12=Vitamin B12) Quantities are given in milligrams per day.

Formulation type	PL		Folate		B12	
	Range mg	Preferred mg	Range mg	Preferred mg	Range mg	Preferred mg
Normal (no absorption problem)	2-5	5	0,2-15	1,0	0.1-2	0.5
Special (to overcome absorption problems)	2-50	5	2-15	5	0.2-5	1,0

One of the mechanisms by which homocysteine causes vascular and other organ pathology is by means of oxidative modification of lipoproteins. It is known that homocysteine potentiates the oxidation of lipoprotein cholesterol with the formation of oxysterols (Biochi. Biogh. Acta 1987, 917:337) and it is also known that oxycholesterol is much more atherogenic than cholesterol itself.

According to yet another aspect of the invention, provision is made to suppress the homocysteine catalyzed oxidation of lipoprotein cholesterol. This may be of benefit to patients with very high homo-

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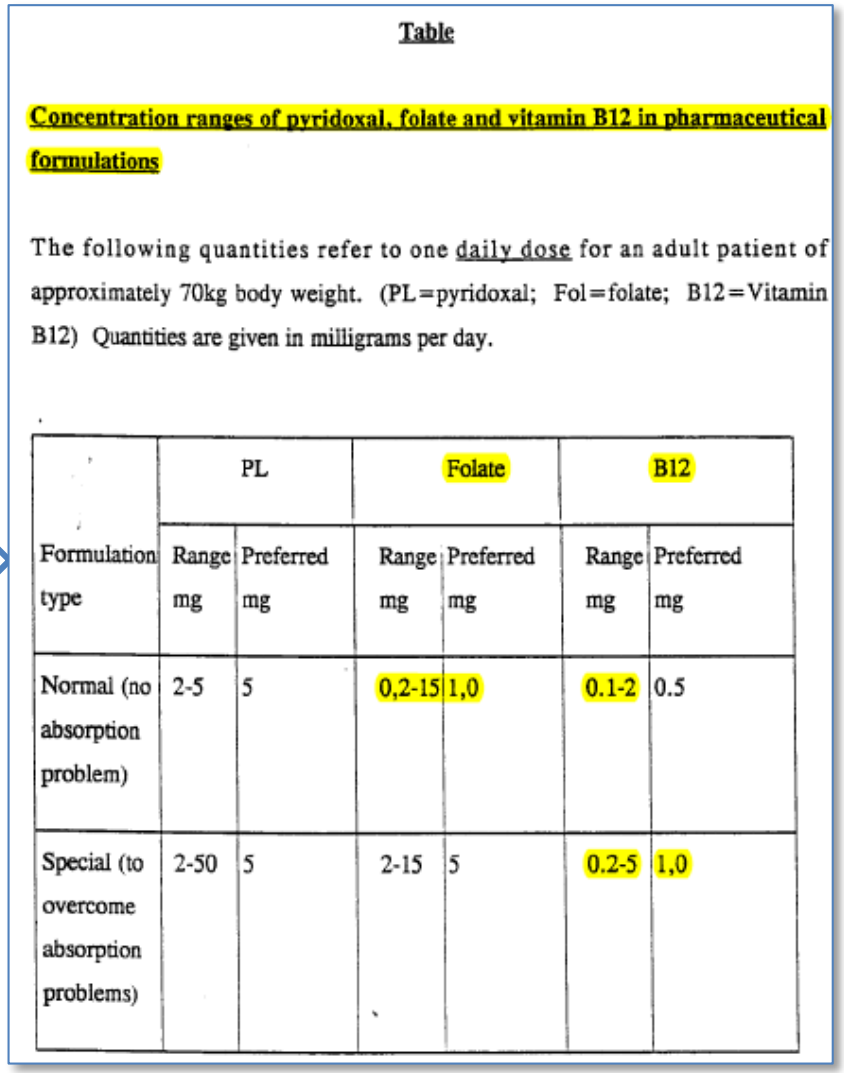
NEPTUNE GENERICS 1010 - 00008



Table

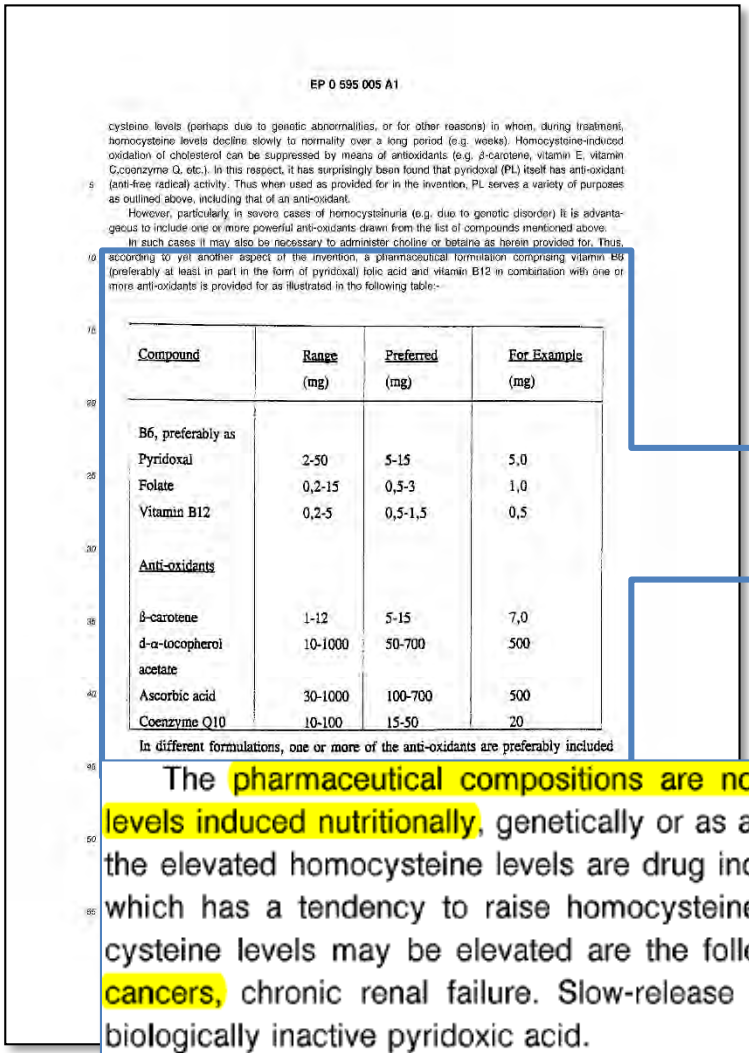
Concentration ranges of pyridoxal, folate and vitamin B12 in pharmaceutical formulations

The following quantities refer to one daily dose for an adult patient of approximately 70kg body weight. (PL=pyridoxal; Fol=folate; B12=Vitamin B12) Quantities are given in milligrams per day.

Formulation type	PL		Folate		B12	
	Range mg	Preferred mg	Range mg	Preferred mg	Range mg	Preferred mg
Normal (no absorption problem)	2-5	5	0,2-15	1,0	0.1-2	0.5
Special (to overcome absorption problems)	2-50	5	2-15	5	0.2-5	1,0

B12 and FA Dosing

EP 005



EP 0 595 005 A1

cysteine levels (perhaps due to genetic abnormalities, or for other reasons) in whom, during treatment, homocysteine levels decline slowly to normality over a long period (e.g. weeks). Homocysteine-induced oxidation of cholesterol can be suppressed by means of antioxidants (e.g. β-carotene, vitamin E, vitamin C,coenzyme Q, etc.). In this respect, it has surprisingly been found that pyridoxal (PL) itself has anti-oxidant (anti-free radical) activity. Thus when used as provided for in the invention, PL serves a variety of purposes as outlined above, including that of an anti-oxidant.

However, particularly in severe cases of homocystsinuria (e.g. due to genetic disorder) it is advantageous to include one or more powerful anti-oxidants drawn from the list of compounds mentioned above.

In such cases it may also be necessary to administer choline or betaine as herein provided for. Thus, according to yet another aspect of the invention, a pharmaceutical formulation comprising vitamin B6 (preferably at least in part in the form of pyridoxal) folic acid and vitamin B12 in combination with one or more anti-oxidants is provided for as illustrated in the following table:-

Compound	Range (mg)	Preferred (mg)	For Example (mg)
B6, preferably as Pyridoxal	2-50	5-15	5,0
Folate	0,2-15	0,5-3	1,0
Vitamin B12	0,2-5	0,5-1,5	0,5
Anti-oxidants			
ß-carotene	1-12	5-15	7,0
d-α-tocopherol acetate	10-1000	50-700	500
Ascorbic acid	30-1000	100-700	500
Coenzyme Q10	10-100	15-50	20

In different formulations, one or more of the anti-oxidants are preferably included

according to yet another aspect of the invention, a pharmaceutical formulation comprising vitamin B6 (preferably at least in part in the form of pyridoxal) folic acid and vitamin B12 in combination with one or more anti-oxidants is provided for as illustrated in the following table:-

Compound	Range (mg)	Preferred (mg)	For Example (mg)
B6, preferably as Pyridoxal	2-50	5-15	5,0
Folate	0,2-15	0,5-3	1,0
Vitamin B12	0,2-5	0,5-1,5	0,5
Anti-oxidants			
ß-carotene	1-12	5-15	7,0
d-α-tocopherol acetate	10-1000	50-700	500
Ascorbic acid	30-1000	100-700	500
Coenzyme Q10	10-100	15-50	20

The pharmaceutical compositions are not only to be used in the treatment of raised homocysteine levels induced nutritionally, genetically or as a result of a variety of diseases, but also in those cases where the elevated homocysteine levels are drug induced or in combination with a B6 or folate antagonistic drug, which has a tendency to raise homocysteine levels. Examples of other situations in which blood homocysteine levels may be elevated are the following: post-menopausal women, liver failure, leukemia, other cancers, chronic renal failure. Slow-release formulation of PL prevents excessive liver oxidation to the biologically inactive pyridoxic acid.

B12 and FA Dosing

EP 005

A pharmaceutical preparation which comprises in combination, each in a concentration and form effective to suppress homocysteine levels in plasma

a) vitamin B6;

b) folate or a suitable active metabolite of folate or a substance which releases folate in vivo;

c) vitamin B12, with or without intrinsic factor,

b):c) from 100:1 to 1:50

4. The invention as claimed in claim 1 or 2, characterised in that the ratios are:
a):b) from 60:1 to 1:1.5
b):c) from 15:1 to 1:2

5. The inventi
a):b) from 2
b):c) from 4
and in parti
a):b) from 2
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facilitate or prescribe to the user the combined administration of the dosage units according to a specific dosage regimen.

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NEPTUNE GENERICS 1010 - 00019

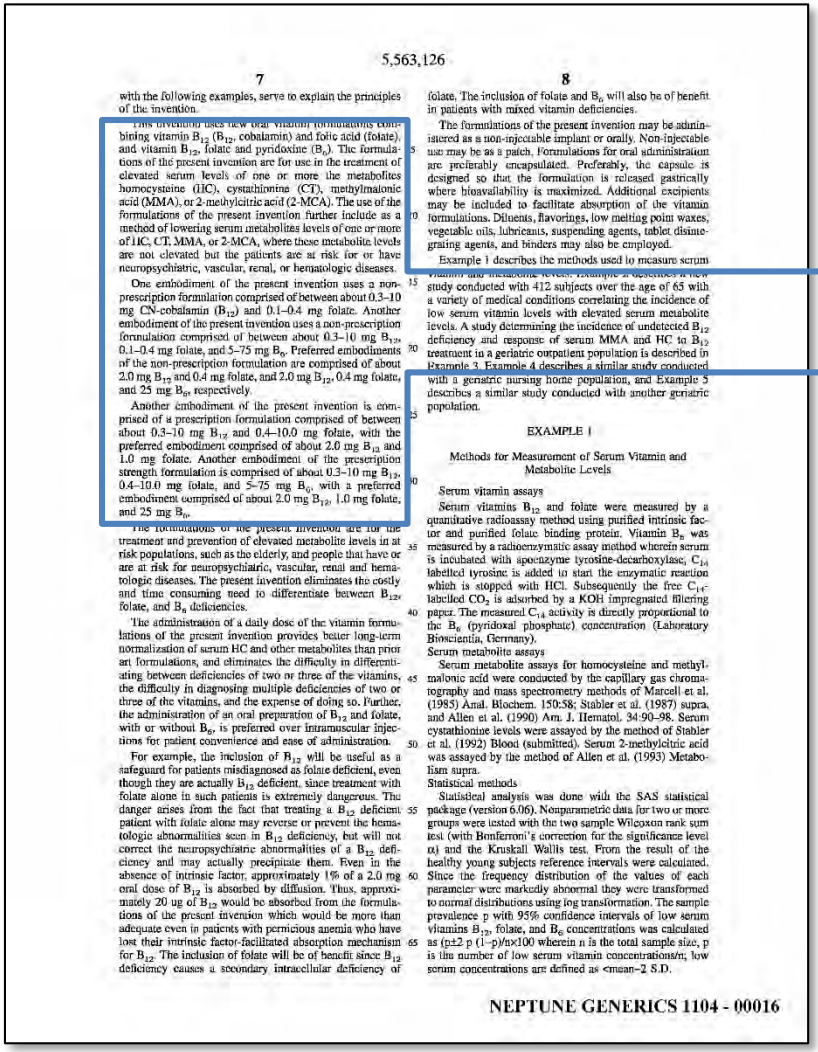
The invention as claimed in any one of claims 1 to 5, characterised in that the preparation is formulated to provide approximate daily dosages as follows (µg/d/kg body weight):

	a) Vitamin B6	b) Folic Acid	c) Vitamin B12
Broadest range	15-750	1,5-150	1,5-75
preferred range	30 - 400	7,5 - 50	3 - 15
more preferred range	75 - 250	10 - 30	7 - 10
typical example	150	15	7,5

Source: Ex. 1010 at claims; Paper 1 at 28, 36, 42.

B12 and FA Dosing

'126 Patent



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with the following examples, serve to explain the principles of the invention.

This invention uses new oral vitamin formulations combining vitamin B_{12} (B_{12}, cobalamin) and folic acid (folate), and vitamin B_{12}, folate and pyridoxine (B_6). The formulations of the present invention are for use in the treatment of elevated serum levels of one or more the metabolites homocysteine (HC), cystathionine (CT), methylmalonic acid (MMA), or 2-methylcitric acid (2-MCA). The use of the formulations of the present invention further include as a method of lowering serum metabolites levels of one or more of HC, CT, MMA, or 2-MCA, where these metabolite levels are not elevated but the patients are at risk for or have neuropsychiatric, vascular, renal, or hematologic diseases.

One embodiment of the present invention uses a non-prescription formulation comprised of between about 0.3–10 mg CN-cobalamin (B_{12}) and 0.1–0.4 mg folate. Another embodiment of the present invention uses a non-prescription formulation comprised of between about 0.3–10 mg B_{12}, 0.1–0.4 mg folate, and 5–75 mg B_6. Preferred embodiments of the non-prescription formulation are comprised of about 2.0 mg B_{12} and 0.4 mg folate, and 2.0 mg B_{12}, 0.4 mg folate, and 25 mg B_6, respectively.

Another embodiment of the present invention is comprised of a prescription formulation comprised of between about 0.3–10 mg B_{12} and 0.4–10.0 mg folate, with the preferred embodiment comprised of about 2.0 mg B_{12} and 1.0 mg folate. Another embodiment of the prescription strength formulation is comprised of about 0.3–10 mg B_{12}, 0.4–10.0 mg folate, and 5–75 mg B_6, with a preferred embodiment comprised of about 2.0 mg B_{12}, 1.0 mg folate, and 25 mg B_6.

The formulations of the present invention are for the treatment and prevention of elevated metabolite levels in at risk populations, such as the elderly, and people that have or are at risk for neuropsychiatric, vascular, renal and hematologic diseases. The present invention eliminates the costly and time consuming need to differentiate between B_{12}, folate, and B_6 deficiencies.

The administration of a daily dose of the vitamin formulations of the present invention provides better long-term normalization of serum HC and other metabolites than prior art formulations, and eliminates the difficulty in differentiating between deficiencies of two or three of the vitamins, the difficulty in diagnosing multiple deficiencies of two or three of the vitamins, and the expense of doing so. Further, the administration of an oral preparation of B_{12} and folate, with or without B_6, is preferred over intramuscular injections for patient convenience and ease of administration.

For example, the inclusion of B_{12} will be useful as a safeguard for patients misdiagnosed as folate deficient, even though they are actually B_{12} deficient, since treatment with folate alone in such patients is extremely dangerous. The danger arises from the fact that treating a B_{12} deficient patient with folate alone may reverse or prevent the hematologic abnormalities seen in B_{12} deficiency, but will not correct the neuropsychiatric abnormalities of a B_{12} deficiency and may actually precipitate them. Even in the absence of intrinsic factor, approximately 1% of a 2.0 mg oral dose of B_{12} is absorbed by diffusion. Thus, approximately 20 ug of B_{12} would be absorbed from the formulations of the present invention which would be more than adequate even in patients with pernicious anemia who have lost their intrinsic factor-facilitated absorption mechanism for B_{12}. The inclusion of folate will be of benefit since B_{12} deficiency causes a secondary intracellular deficiency of folate. The inclusion of folate and B_6 will also be of benefit in patients with mixed vitamin deficiencies.

The formulations of the present invention may be administered as a non-injectable implant or orally. Non-injectable use may be as a patch. Formulations for oral administration are preferably encapsulated. Preferably, the capsule is designed so that the formulation is released gastrically where bioavailability is maximized. Additional excipients may be included to facilitate absorption of the vitamin formulations. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.

Example 1 describes the methods used to measure serum vitamin and metabolic levels. Example 2 describes a new study conducted with 412 subjects over the age of 65 with a variety of medical conditions correlating the incidence of low serum vitamin levels with elevated serum metabolite levels. A study determining the incidence of undetected B_{12} deficiency and response of serum MMA and HC to B_{12} treatment in a geriatric outpatient population is described in Example 3. Example 4 describes a similar study conducted with a geriatric nursing home population, and Example 5 describes a similar study conducted with another geriatric population.

EXAMPLE 1

Methods for Measurement of Serum Vitamin and Metabolite Levels

Serum vitamin assays

Serum vitamins B_{12} and folate were measured by a quantitative radioassay method using purified intrinsic factor and purified folate binding protein. Vitamin B_6 was measured by a radioenzymatic assay method wherein serum is incubated with apoenzyme tyrosine-decarboxylase, C_{14} labelled tyrosine is added to start the enzymatic reaction which is stopped with HCl. Subsequently the free C_{14}-labelled CO_2 is absorbed by a KOH impregnated filtering paper. The measured C_{14} activity is directly proportional to the B_6 (pyridoxal phosphate) concentration (Laboratory Biosciential, Germany).

Serum metabolite assays

Serum metabolite assays for homocysteine and methylmalonic acid were conducted by the capillary gas chromatography and mass spectrometry methods of Marcell et al. (1985) Anal. Biochem. 150:58; Stabler et al. (1987) supra, and Allen et al. (1990) Am. J. Hematol. 34:90–98. Serum cystathionine levels were assayed by the method of Stabler et al. (1992) Blood (submitted). Serum 2-methylcitric acid was assayed by the method of Allen et al. (1993) Metabolism supra.

Statistical methods

Statistical analysis was done with the SAS statistical package (version 6.06). Nonparametric data for two or more groups were tested with the two sample Wilcoxon rank sum test (with Bonferroni's correction for the significance level α) and the Kruskall Wallis test. From the result of the healthy young subjects reference intervals were calculated. Since the frequency distribution of the values of each parameter were markedly abnormal they were transformed to normal distributions using log transformation. The sample prevalence p with 95% confidence intervals of low serum vitamins B_{12}, folate, and B_6 concentrations was calculated as $(p \pm 2\sqrt{p(1-p)/n} \times 100$ wherein n is the total sample size, p is the number of low serum vitamin concentrations/n, low serum concentrations are defined as <mean–2 S.D.

NEPTUNE GENERICS 1104 - 00016

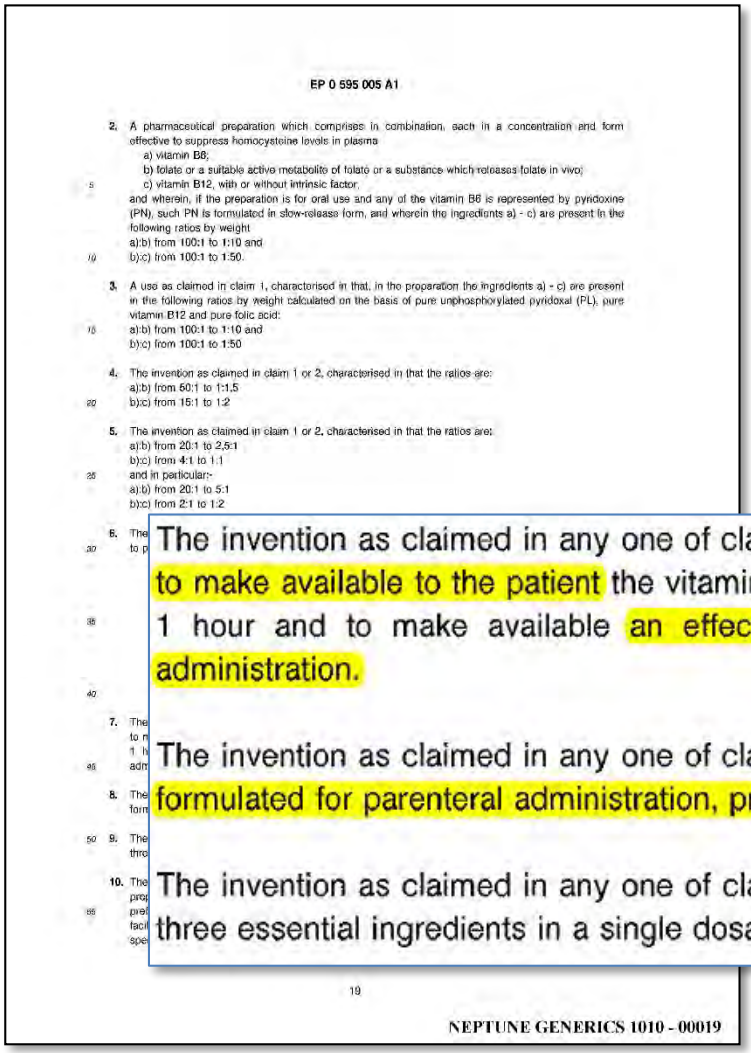
This invention uses new oral vitamin formulations combining vitamin B_{12} (B_{12}, cobalamin) and folic acid (folate), and vitamin B_{12}, folate and pyridoxine (B_6). The formulations of the present invention are for use in the treatment of elevated serum levels of one or more the metabolites homocysteine (HC), cystathionine (CT), methylmalonic acid (MMA), or 2-methylcitric acid (2-MCA). The use of the formulations of the present invention further include as a method of lowering serum metabolites levels of one or more of HC, CT, MMA, or 2-MCA, where these metabolite levels are not elevated but the patients are at risk for or have neuropsychiatric, vascular, renal, or hematologic diseases.

One embodiment of the present invention uses a non-prescription formulation comprised of between about 0.3–10 mg CN-cobalamin (B_{12}) and 0.1–0.4 mg folate. Another embodiment of the present invention uses a non-prescription formulation comprised of between about 0.3–10 mg B_{12}, 0.1–0.4 mg folate, and 5–75 mg B_6. Preferred embodiments of the non-prescription formulation are comprised of about 2.0 mg B_{12} and 0.4 mg folate, and 2.0 mg B_{12}, 0.4 mg folate, and 25 mg B_6, respectively.

Another embodiment of the present invention is comprised of a prescription formulation comprised of between about 0.3–10 mg B_{12} and 0.4–10.0 mg folate, with the preferred embodiment comprised of about 2.0 mg B_{12} and 1.0 mg folate. Another embodiment of the prescription strength formulation is comprised of about 0.3–10 mg B_{12}, 0.4–10.0 mg folate, and 5–75 mg B_6, with a preferred embodiment comprised of about 2.0 mg B_{12}, 1.0 mg folate, and 25 mg B_6.

B12 Dosing

EP 005



EP 0 595 005 A1

2. A pharmaceutical preparation which comprises in combination, each in a concentration and form effective to suppress homocysteine levels in plasma
   a) vitamin B6;
   b) folate or a suitable active metabolite of folate or a substance which releases folate in vivo;
   c) vitamin B12, with or without intrinsic factor,
   and wherein, if the preparation is for oral use and any of the vitamin B6 is represented by pyridoxine (PN), such PN is formulated in slow-release form, and wherein the ingredients a) - c) are present in the following ratios by weight
   a):b) from 100:1 to 1:10 and
   b):c) from 100:1 to 1:50.

3. A use as claimed in claim 1, characterised in that, in the preparation the ingredients a) - c) are present in the following ratios by weight calculated on the basis of pure unphosphorylated pyridoxal (PL), pure vitamin B12 and pure folic acid:
   a):b) from 100:1 to 1:10 and
   b):c) from 100:1 to 1:50

4. The invention as claimed in claim 1 or 2, characterised in that the ratios are:
   a):b) from 60:1 to 1:1.5
   b):c) from 15:1 to 1:2

5. The invention as claimed in claim 1 or 2, characterised in that the ratios are:
   a):b) from 20:1 to 2,5:1
   b):c) from 4:1 to 1:1
   and in particular:-
   a):b) from 20:1 to 5:1
   b):c) from 2:1 to 1:2

6. The invention as claimed in any one of claims 1 to 6, characterised in that the preparation is formulated to make available to the patient the vitamin B6 and preferably also the folate over a period of more than 1 hour and to make available an effective dosage of the vitamin B12 in less than 1 hour after administration.

7. The invention as claimed in any one of claims 1 to 7, characterised in that the preparation is galenically formulated for parenteral administration, preferably by infusion or by intramuscular injection.

8. The invention as claimed in any one of claims 1 to 8, characterised in that the preparation combines all three essential ingredients in a single dosage form, preferably designed for oral administration.

9. The

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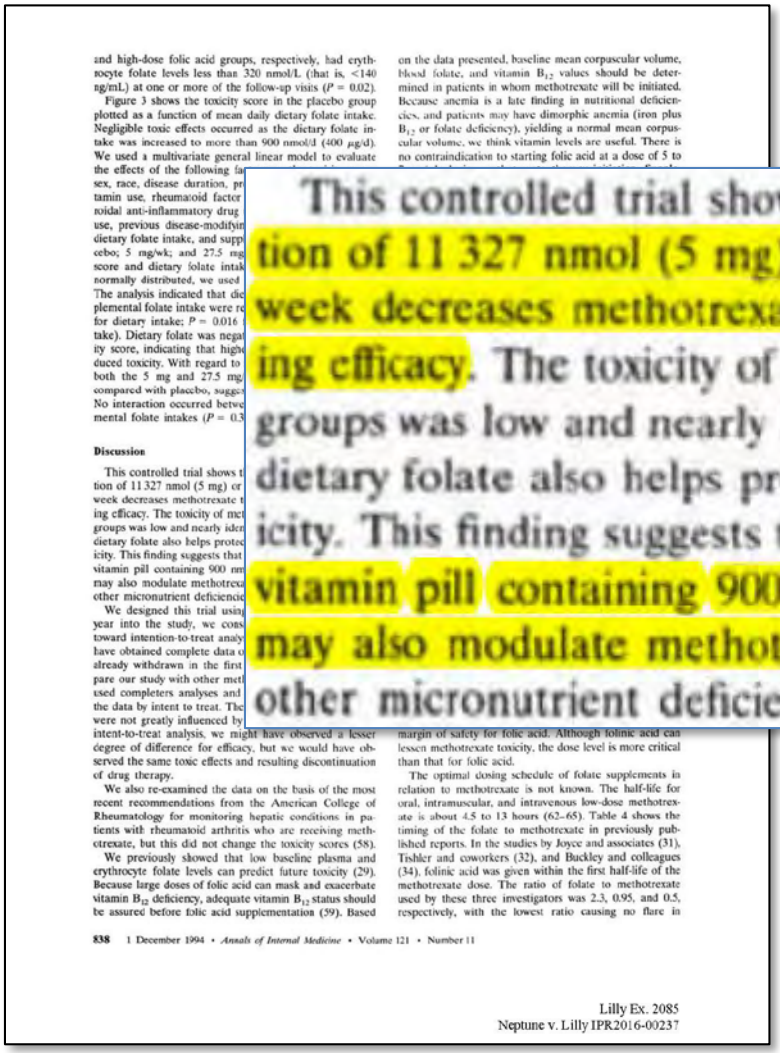
NEPTUNE GENERICS 1010 - 00019

Source: Ex. 1010 at claims; Paper 1 at 28, 36, 42-44, 47.

FA Dosing

Morgan



and high-dose folic acid groups, respectively, had erythrocyte folate levels less than 320 nmol/L (that is, <140 ng/mL) at one or more of the follow-up visits ($P = 0.02$).

Figure 3 shows the toxicity score in the placebo group plotted as a function of mean daily dietary folate intake. Negligible toxic effects occurred as the dietary folate intake was increased to more than 900 nmol/d (400 μg/d). We used a multivariate general linear model to evaluate the effects of the following fa… sex, race, disease duration, pr… tamin use, rheumatoid factor … noidal anti-inflammatory drug … use, previous disease-modifyi… dietary folate intake, and supp… cebo; 5 mg/wk; and 27.5 mg… score and dietary folate intak… normally distributed, we used … The analysis indicated that die… plemental folate intake were r… for dietary intake; $P = 0.016$ … take). Dietary folate was nega… ity score, indicating that high… duced toxicity. With regard to … both the 5 mg and 27.5 mg… compared with placebo, sugg… No interaction occurred betwe… mental folate intakes ($P = 0.$…

Discussion

This controlled trial shows t… tion of 11 327 nmol (5 mg) or … week decreases methotrexate t… ing efficacy. The toxicity of met… groups was low and nearly iden… dietary folate also helps protec… icity. This finding suggests that … vitamin pill containing 900 nm… may also modulate methotrexa… other micronutrient deficiencie…

We designed this trial using … year into the study, we cons… toward intention-to-treat analy… have obtained complete data o… already withdrawn in the first … pare our study with other met… used completers analyses and … the data by intent to treat. The … were not greatly influenced by … intent-to-treat analysis, we might have observed a lesser degree of difference for efficacy, but we would have observed the same toxic effects and resulting discontinuation of drug therapy.

We also re-examined the data on the basis of the most recent recommendations from the American College of Rheumatology for monitoring hepatic conditions in patients with rheumatoid arthritis who are receiving methotrexate, but this did not change the toxicity scores (58).

We previously showed that low baseline plasma and erythrocyte folate levels can predict future toxicity (29). Because large doses of folic acid can mask and exacerbate vitamin B_{12} deficiency, adequate vitamin B_{12} status should be assured before folic acid supplementation (59). Based on the data presented, baseline mean corpuscular volume, blood folate, and vitamin B_{12} values should be determined in patients in whom methotrexate will be initiated. Because anemia is a late finding in nutritional deficiencies, and patients may have dimorphic anemia (iron plus B_{12} or folate deficiency), yielding a normal mean corpuscular volume, we think vitamin levels are useful. There is no contraindication to starting folic acid at a dose of 5 to …

margin of safety for folic acid. Although folinic acid can lessen methotrexate toxicity, the dose level is more critical than that for folic acid.

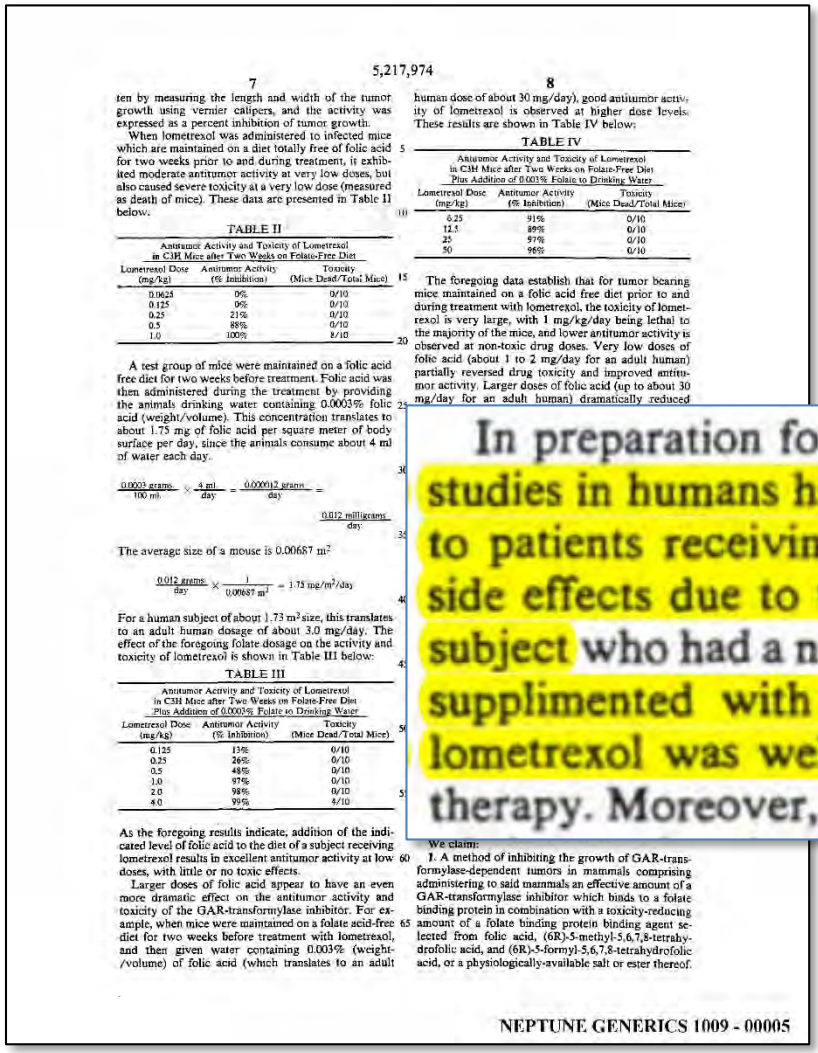
The optimal dosing schedule of folate supplements in relation to methotrexate is not known. The half-life for oral, intramuscular, and intravenous low-dose methotrexate is about 4.5 to 13 hours (62–65). Table 4 shows the timing of the folate to methotrexate in previously published reports. In the studies by Joyce and associates (31), Tishler and coworkers (32), and Buckley and colleagues (34), folinic acid was given within the first half-life of the methotrexate dose. The ratio of folate to methotrexate used by these three investigators was 2.3, 0.95, and 0.5, respectively, with the lowest ratio causing no flare in

> This controlled trial shows that folic acid supplementation of 11 327 nmol (5 mg) or 62 302 nmol (27.5 mg) per week decreases methotrexate toxicity without compromising efficacy. The toxicity of methotrexate in both folic acid groups was low and nearly identical. The data suggest that dietary folate also helps protect against methotrexate toxicity. This finding suggests that the intake of one multiple-vitamin pill containing 900 nmol of folic acid (400 μg/d) may also modulate methotrexate toxicity in patients with other micronutrient deficiencies (57).

Source: Ex. 2085 at 838; Paper 48 at 30.

FA Dosing

’974 Patent



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ten by measuring the length and width of the tumor growth using vernier calipers, and the activity was expressed as a percent inhibition of tumor growth.

When lometrexol was administered to infected mice which are maintained on a diet totally free of folic acid for two weeks prior to and during treatment, it exhibited moderate antitumor activity at very low doses, but also caused severe toxicity at a very low dose (measured as death of mice). These data are presented in Table II below.

TABLE II

Antitumor Activity and Toxicity of Lometrexol in C3H Mice after Two Weeks on Folate-Free Diet

Lometrexol Dose (mg/kg)	Antitumor Activity (% Inhibition)	Toxicity (Mice Dead/Total Mice)
0.0625	0%	0/10
0.125	0%	0/10
0.25	21%	0/10
0.5	88%	0/10
1.0	100%	8/10

A test group of mice were maintained on a folic acid free diet for two weeks before treatment. Folic acid was then administered during the treatment by providing the animals drinking water containing 0.0003% folic acid (weight/volume). This concentration translates to about 1.75 mg of folic acid per square meter of body surface per day, since the animals consume about 4 ml of water each day.

$$\frac{0.0003 \text{ grams}}{100 \text{ ml}} \times \frac{4 \text{ ml}}{\text{day}} = \frac{0.000012 \text{ grams}}{\text{day}} = \frac{0.012 \text{ milligrams}}{\text{day}}$$

The average size of a mouse is 0.00687 m^2

$$\frac{0.012 \text{ grams}}{\text{day}} \times \frac{1}{0.00687 \text{ m}^2} = 1.75 \text{ mg/m}^2\text{/day}$$

For a human subject of about 1.73 m^2 size, this translates to an adult human dosage of about 3.0 mg/day. The effect of the foregoing folate dosage on the activity and toxicity of lometrexol is shown in Table III below:

TABLE III

Antitumor Activity and Toxicity of Lometrexol in C3H Mice after Two Weeks on Folate-Free Diet Plus Addition of 0.003% Folate to Drinking Water

Lometrexol Dose (mg/kg)	Antitumor Activity (% Inhibition)	Toxicity (Mice Dead/Total Mice)
0.125	13%	0/10
0.25	26%	0/10
0.5	48%	0/10
1.0	97%	0/10
2.0	98%	0/10
4.0	99%	4/10

As the foregoing results indicate, addition of the indicated level of folic acid to the diet of a subject receiving lometrexol results in excellent antitumor activity at low doses, with little or no toxic effects.

Larger doses of folic acid appear to have an even more dramatic effect on the antitumor activity and toxicity of the GAR-transformylase inhibitor. For example, when mice were maintained on a folate acid-free diet for two weeks before treatment with lometrexol, and then given water containing 0.003% (weight-/volume) of folic acid (which translates to an adult human dose of about 30 mg/day), good antitumor activity of lometrexol is observed at higher dose levels. These results are shown in Table IV below:

TABLE IV

Antitumor Activity and Toxicity of Lometrexol in C3H Mice after Two Weeks on Folate-Free Diet Plus Addition of 0.003% Folate to Drinking Water

Lometrexol Dose (mg/kg)	Antitumor Activity (% Inhibition)	Toxicity (Mice Dead/Total Mice)
0.25	91%	0/10
12.5	89%	0/10
25	97%	0/10
50	96%	0/10

The foregoing data establish that for tumor bearing mice maintained on a folic acid free diet prior to and during treatment with lometrexol, the toxicity of lometrexol is very large, with 1 mg/kg/day being lethal to the majority of the mice, and lower antitumor activity is observed at non-toxic drug doses. Very low doses of folic acid (about 1 to 2 mg/day for an adult human) partially reversed drug toxicity and improved antitumor activity. Larger doses of folic acid (up to about 30 mg/day for an adult human) dramatically reduced

We claim:

1. A method of inhibiting the growth of GAR-transformylase-dependent tumors in mammals comprising administering to said mammals an effective amount of a GAR-transformylase inhibitor which binds to a folate binding protein in combination with a toxicity-reducing amount of a folate binding protein binding agent selected from folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, and (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or a physiologically-available salt or ester thereof.

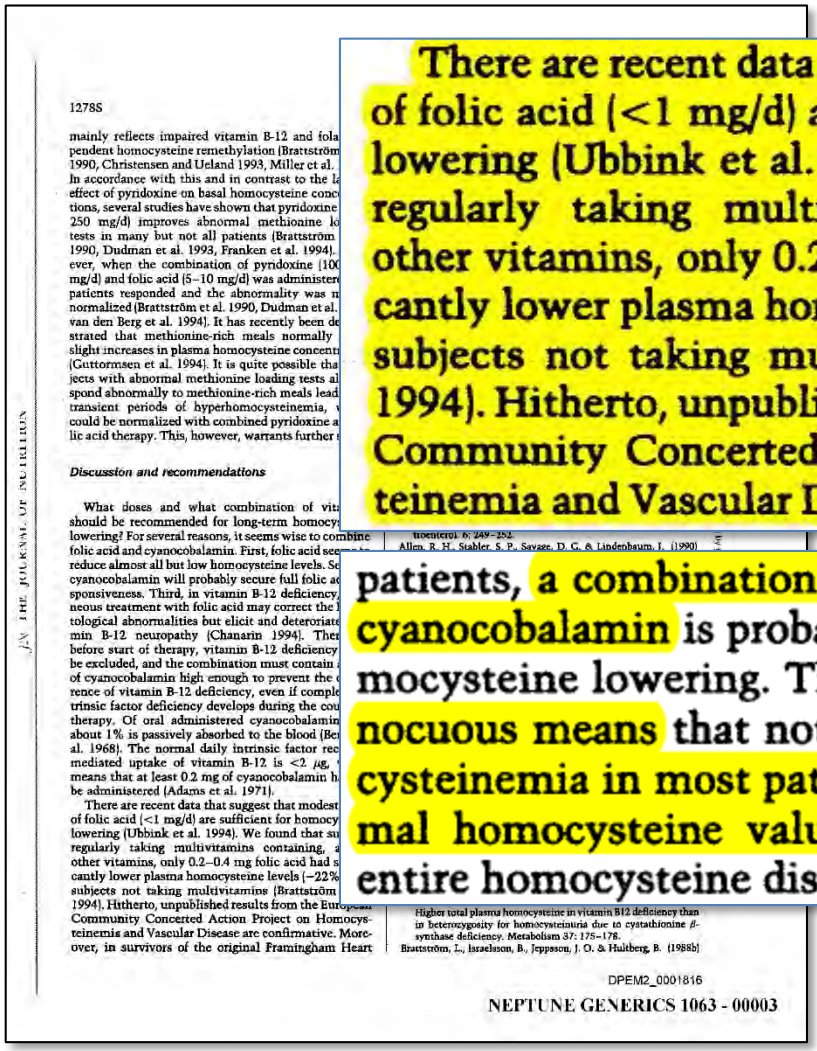
NEPTUNE GENERICS 1009 - 00005

In preparation for the foregoing clinical study, pilot studies in humans have established that folic acid given to patients receiving lometrexol has effected reduced side effects due to the lometrexol. Specifically, in one subject who had a nasalpharyngeal carcinoma, who was supplimented with folic acid at 0.5 to 1.0 mg/day, lometrexol was well tolerated for up to 12 months of therapy. Moreover, this patient has no clinical evidence

Source: Ex. 1009 at 8:49-56; Paper 48 at 30.

FA Dosing

Brattström



1278S

mainly reflects impaired vitamin B-12 and fola
pendent homocysteine remethylation (Brattström
1990, Christensen and Ueland 1993, Miller et al.
In accordance with this and in contrast to the la
effect of pyridoxine on basal homocysteine conc
tions, several studies have shown that pyridoxine
250 mg/d) improves abnormal methionine lo
tests in many but not all patients (Brattström
1990, Dudman et al. 1993, Franken et al. 1994).
ever, when the combination of pyridoxine (10
mg/d) and folic acid (5–10 mg/d) was administer
patients responded and the abnormality was n
normalized (Brattström et al. 1990, Dudman et al.
van den Berg et al. 1994). It has recently been de
strated that methionine-rich meals normally
slight increases in plasma homocysteine concentr
(Guttormsen et al. 1994). It is quite possible tha
jects with abnormal methionine loading tests al
spond abnormally to methionine-rich meals lead
transient periods of hyperhomocysteinemia,
could be normalized with combined pyridoxine a
lic acid therapy. This, however, warrants further

Discussion and recommendations

What doses and what combination of vita
should be recommended for long-term homocys
lowering? For several reasons, it seems wise to combine
folic acid and cyanocobalamin. First, folic acid se
reduce almost all but low homocysteine levels. Se
cyanocobalamin will probably secure full folic ac
sponsiveness. Third, in vitamin B-12 deficiency,
neous treatment with folic acid may correct the l
tological abnormalities but elicit and deteroriate
min B-12 neuropathy (Chanarin 1994). Ther
before start of therapy, vitamin B-12 deficiency
be excluded, and the combination must contain
of cyanocobalamin high enough to prevent the
rence of vitamin B-12 deficiency, even if comple
trinsic factor deficiency develops during the cou
therapy. Of oral administered cyanocobalamin
about 1% is passively absorbed to the blood (Be
al. 1968). The normal daily intrinsic factor rec
mediated uptake of vitamin B-12 is <2 μg,
means that at least 0.2 mg of cyanocobalamin h
be administered (Adams et al. 1971).

There are recent data that suggest that modest
of folic acid (<1 mg/d) are sufficient for homocy
lowering (Ubbink et al. 1994). We found that su
regularly taking multivitamins containing,
other vitamins, only 0.2–0.4 mg folic acid had s
cantly lower plasma homocysteine levels (−22%
subjects not taking multivitamins (Brattström
1994). Hitherto, unpublished results from the Eur
Community Concerted Action Project on Homocys-
teinemia and Vascular Disease are confirmative. More-
over, in survivors of the original Framingham Heart

tocentrol. 6: 249–252.
Allen, R. H., Stabler, S. P., Savage, D. G. & Lindenbaum, J. (1990)

Higher total plasma homocysteine in vitamin B12 deficiency than in heterozygosity for homocystinuria due to cystathionine β-synthase deficiency. Metabolism 37: 175–178.
Brattström, L., Israelsson, B., Jeppsson, J. O. & Hultberg, B. (1988b)

DPEM2_0001816

NEPTUNE GENERICS 1063 - 00003

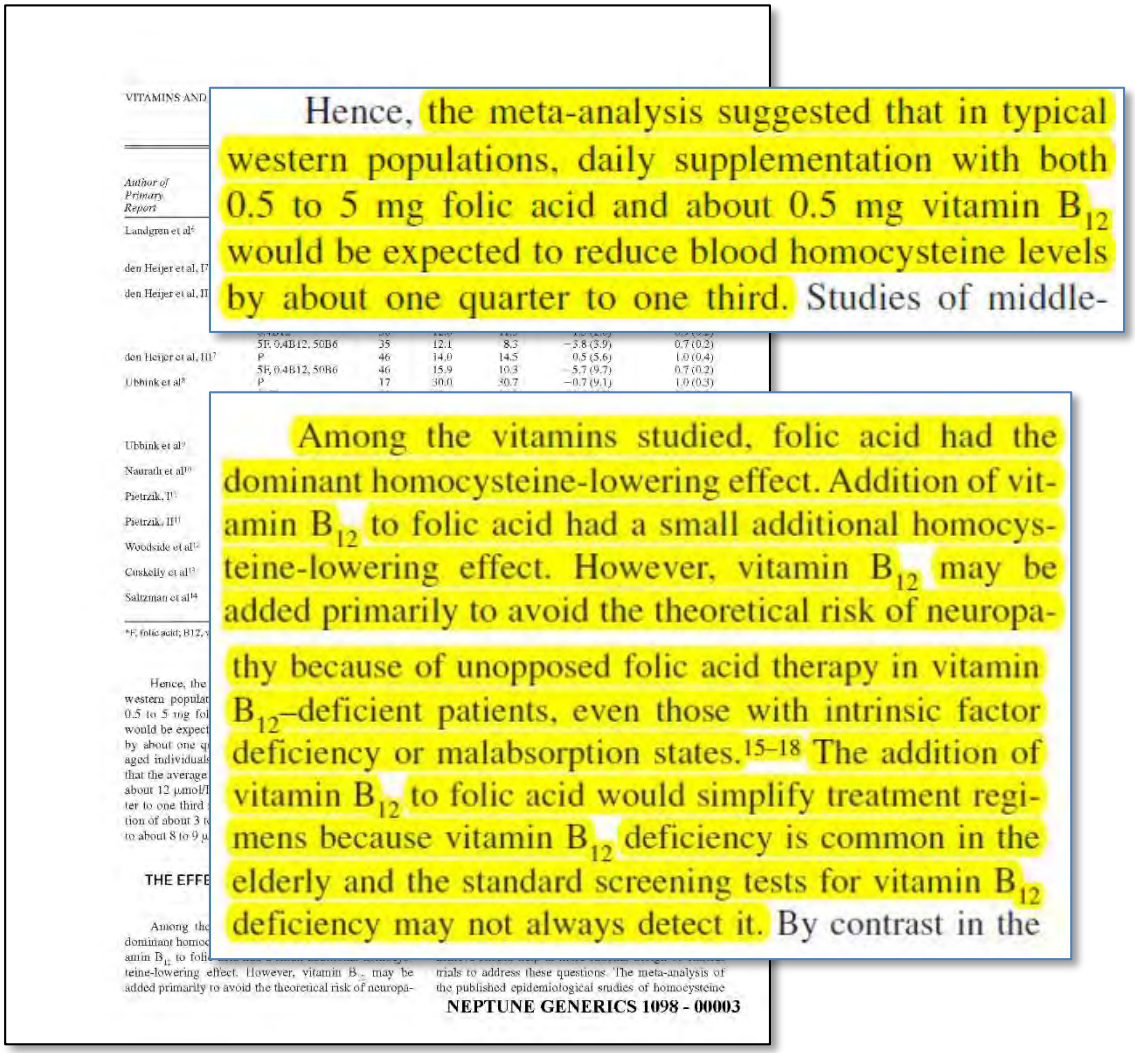
There are recent data that suggest that modest doses of folic acid (<1 mg/d) are sufficient for homocysteine lowering (Ubbink et al. 1994). We found that subjects regularly taking multivitamins containing, among other vitamins, only 0.2–0.4 mg folic acid had significantly lower plasma homocysteine levels (−22%) than subjects not taking multivitamins (Brattström et al. 1994). Hitherto, unpublished results from the European Community Concerted Action Project on Homocysteinemia and Vascular Disease are confirmative. More-

patients, a combination of 1 mg folic acid and 0.4 mg cyanocobalamin is probably sufficient for effective homocysteine lowering. This combination will be an innocuous means that not only normalizes hyperhomocysteinemia in most patients but also will reduce normal homocysteine values, leading to a shift of the entire homocysteine distribution toward lower values.

Source: Ex. 1063 at 1278S; Paper 48 at 21.

FA Dosing

Clarke



Hence, the meta-analysis suggested that in typical western populations, daily supplementation with both 0.5 to 5 mg folic acid and about 0.5 mg vitamin B_{12} would be expected to reduce blood homocysteine levels by about one quarter to one third. Studies of middle-

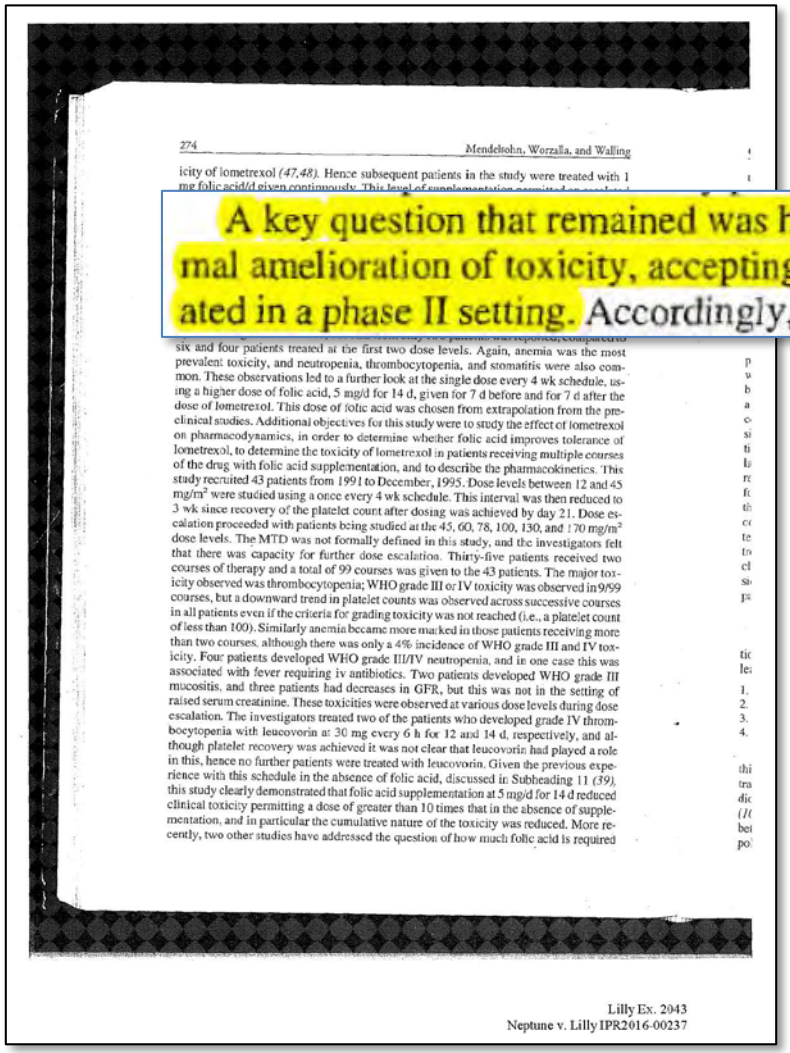
Among the vitamins studied, folic acid had the dominant homocysteine-lowering effect. Addition of vitamin B_{12} to folic acid had a small additional homocysteine-lowering effect. However, vitamin B_{12} may be added primarily to avoid the theoretical risk of neuropathy because of unopposed folic acid therapy in vitamin B_{12}–deficient patients, even those with intrinsic factor deficiency or malabsorption states.[15–18] The addition of vitamin B_{12} to folic acid would simplify treatment regimens because vitamin B_{12} deficiency is common in the elderly and the standard screening tests for vitamin B_{12} deficiency may not always detect it. By contrast in the

NEPTUNE GENERICS 1098 - 00003

Source: Ex. 1098 at 343; Paper 48 at 20.

FA Dosing

Mendelsohn



274 Mendelsohn, Worzalla, and Walling

icity of lometrexol (47,48). Hence subsequent patients in the study were treated with 1 mg folic acid/d given continuously. This level of supplementation permitted an escalation

six and four patients treated at the first two dose levels. Again, anemia was the most prevalent toxicity, and neutropenia, thrombocytopenia, and stomatitis were also common. These observations led to a further look at the single dose every 4 wk schedule, using a higher dose of folic acid, 5 mg/d for 14 d, given for 7 d before and for 7 d after the dose of lometrexol. This dose of folic acid was chosen from extrapolation from the preclinical studies. Additional objectives for this study were to study the effect of lometrexol on pharmacodynamics, in order to determine whether folic acid improves tolerance of lometrexol, to determine the toxicity of lometrexol in patients receiving multiple courses of the drug with folic acid supplementation, and to describe the pharmacokinetics. This study recruited 43 patients from 1991 to December, 1995. Dose levels between 12 and 45 mg/m^2 were studied using a once every 4 wk schedule. This interval was then reduced to 3 wk since recovery of the platelet count after dosing was achieved by day 21. Dose escalation proceeded with patients being studied at the 45, 60, 78, 100, 130, and 170 mg/m^2 dose levels. The MTD was not formally defined in this study, and the investigators felt that there was capacity for further dose escalation. Thirty-five patients received two courses of therapy and a total of 99 courses was given to the 43 patients. The major toxicity observed was thrombocytopenia; WHO grade III or IV toxicity was observed in 9/99 courses, but a downward trend in platelet counts was observed across successive courses in all patients even if the criteria for grading toxicity was not reached (i.e., a platelet count of less than 100). Similarly anemia became more marked in those patients receiving more than two courses, although there was only a 4% incidence of WHO grade III and IV toxicity. Four patients developed WHO grade III/IV neutropenia, and in one case this was associated with fever requiring iv antibiotics. Two patients developed WHO grade III mucositis, and three patients had decreases in GFR, but this was not in the setting of raised serum creatinine. These toxicities were observed at various dose levels during dose escalation. The investigators treated two of the patients who developed grade IV thrombocytopenia with leucovorin at 30 mg every 6 h for 12 and 14 d, respectively, and although platelet recovery was achieved it was not clear that leucovorin had played a role in this, hence no further patients were treated with leucovorin. Given the previous experience with this schedule in the absence of folic acid, discussed in Subheading 11 (39), this study clearly demonstrated that folic acid supplementation at 5 mg/d for 14 d reduced clinical toxicity permitting a dose of greater than 10 times that in the absence of supplementation, and in particular the cumulative nature of the toxicity was reduced. More recently, two other studies have addressed the question of how much folic acid is required

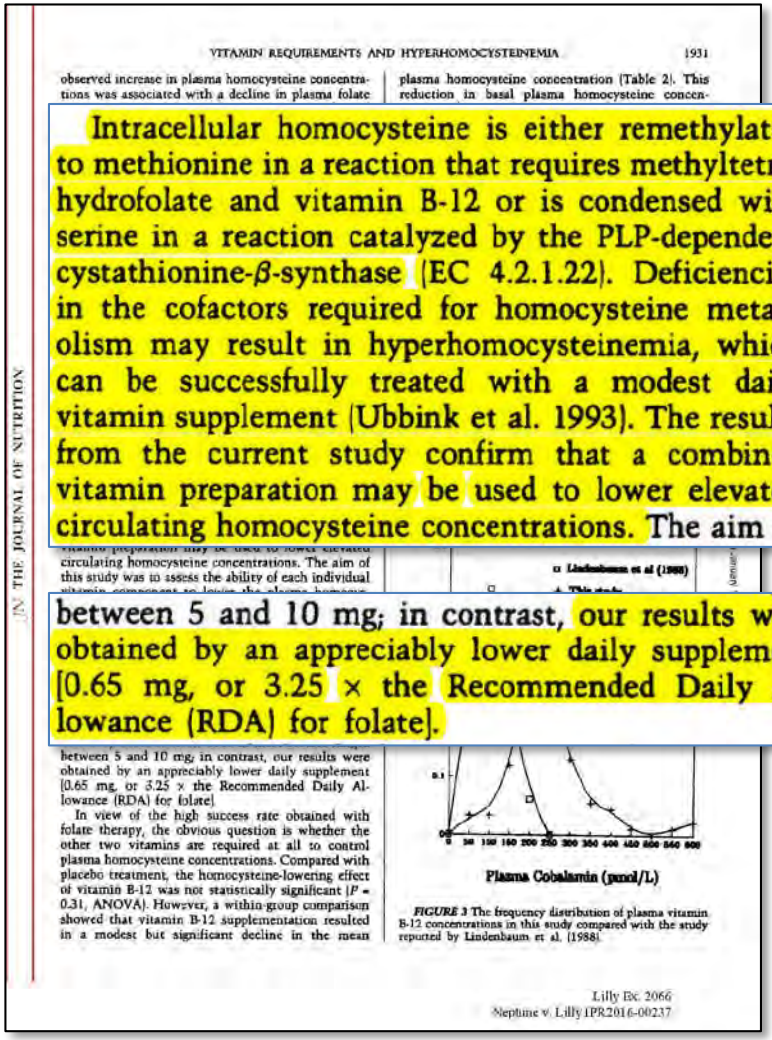
A key question that remained was how much folic acid was required to achieve optimal amelioration of toxicity, accepting that any effects on efficacy could only be evaluated in a phase II setting. Accordingly, a study of a weekly × 3 schedule was conducted

Lilly Ex. 2043
Neptune v. Lilly IPR2016-00237

Source: Ex. 2043 at 274; Paper 48 at 16.

FA Dosing

Ubbink I



VITAMIN REQUIREMENTS AND HYPERHOMOCYSTEINEMIA 1931

observed increase in plasma homocysteine concentrations was associated with a decline in plasma folate

plasma homocysteine concentration (Table 2). This reduction in basal plasma homocysteine concen-

Intracellular homocysteine is either remethylated to methionine in a reaction that requires methyltetrahydrofolate and vitamin B-12 or is condensed with serine in a reaction catalyzed by the PLP-dependent cystathionine-β-synthase (EC 4.2.1.22). Deficiencies in the cofactors required for homocysteine metabolism may result in hyperhomocysteinemia, which can be successfully treated with a modest daily vitamin supplement (Ubbink et al. 1993). The results from the current study confirm that a combined vitamin preparation may be used to lower elevated circulating homocysteine concentrations. The aim of

between 5 and 10 mg; in contrast, our results were obtained by an appreciably lower daily supplement [0.65 mg, or 3.25 × the Recommended Daily Allowance (RDA) for folate].

In view of the high success rate obtained with folate therapy, the obvious question is whether the other two vitamins are required at all to control plasma homocysteine concentrations. Compared with placebo treatment, the homocysteine-lowering effect of vitamin B-12 was not statistically significant ($P = 0.31$, ANOVA). However, a within-group comparison showed that vitamin B-12 supplementation resulted in a modest but significant decline in the mean

Plasma Cobalamin (pmol/L)

FIGURE 3 The frequency distribution of plasma vitamin B-12 concentrations in this study compared with the study reported by Lindenbaum et al. (1988).

Lilly Ex. 2066
Neptune v. Lilly IPR2016-00237

showed that vitamin B-12 supplementation resulted in a modest but significant decline in the mean plasma homocysteine concentration (Table 2). This

min, whereas in our study the hyperhomocysteinemic men were randomized into the different treatment groups without prior knowledge of vitamin nutritional status or any possible genetic aberrations. The

Folic acid supplementation in patients with a chronic vitamin B-12 deficiency may eventually result in neuropathy due to failure to recognize the vitamin B-12 deficiency (Beck 1991). Moreover, Allen et al. (1990) have recently shown that folate supplementation will not correct hyperhomocysteinemia that is primarily the result of a vitamin B-12 deficiency. It is therefore essential that vitamin B-12 and folate be combined to treat hyperhomocysteinemia.

Source: Ex. 2066 at 1931; Paper 48 at 20.

B12 and FA Dose and Schedule

EP 005

The preparation according to the invention was formulated as follows (per oral dosage unit):-

a)	(i) (ii)	pyridoxal pyridoxine	2 mg 8 mg; (i) + (ii) = 10 mg
b)		folate	0,65 mg
c)		cyanocobalamin	0,4 mg.

The following is a summary of results at the beginning of the trial and after 3 weeks. (Results shown only of 78 patients who completed the trial.)

EP 0 595 005 A1

RESULTS

	Placebo (n = 16)	B12 (n = 15)	B6 (n = 17)	folate (n = 19)	Invention (n = 11)
P-HC**) (before)	25,6	23,5	29,8	28,7	27,6
P-HC (after)	26	21,6	28,7	16,4	12,1
%change	+ 1,5	-8,1	-3,7	-42,3	-56,2
p	NS	NS	NS	<0,001	<0,001
%patients	1/16	3/15	3/17	11/19	10/11
normalised	6,3%	20%	17,6%	58%	91% *)

100 hyperhomocysteinemics (P-HC >16,3 µmol/l)

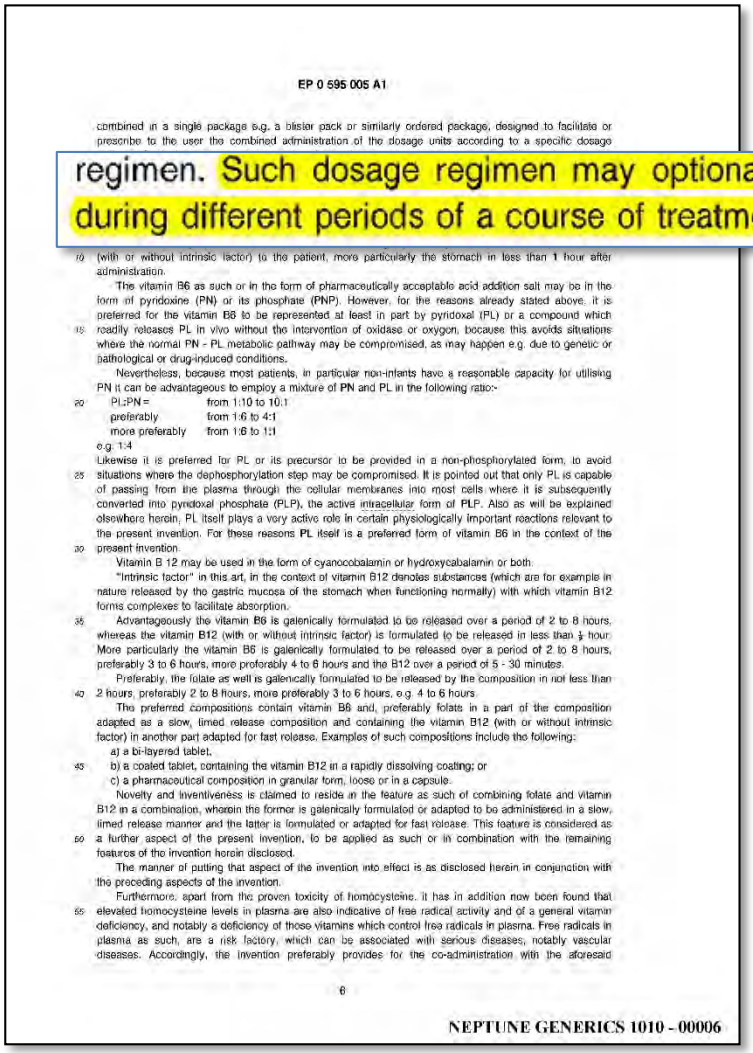
*) reduced 100%

**) Plasma homocysteine

Source: Ex. 1010 at 17-18; Paper 1 at 47; Paper 1 at 47; Paper 48 at 21.

B12 and FA Schedule

EP 005



EP 0 595 005 A1

combined in a single package e.g. a blister pack or similarly ordered package, designed to facilitate or prescribe to the user the combined administration of the dosage units according to a specific dosage regimen. Such dosage regimen may optionally be time programmed, providing for different dosage rates during different periods of a course of treatment. Packages designed for that purpose are known per se and

(with or without intrinsic factor) to the patient, more particularly the stomach in less than 1 hour after administration.

The vitamin B6 as such or in the form of pharmaceutically acceptable acid addition salt may be in the form of pyridoxine (PN) or its phosphate (PNP). However, for the reasons already stated above, it is preferred for the vitamin B6 to be represented at least in part by pyridoxal (PL) or a compound which readily releases PL in vivo without the intervention of oxidase or oxygen, because this avoids situations where the normal PN - PL metabolic pathway may be compromised, as may happen e.g. due to genetic or pathological or drug-induced conditions.

Nevertheless, because most patients, in particular non-infants have a reasonable capacity for utilising PN it can be advantageous to employ a mixture of PN and PL in the following ratio:-

PL:PN = from 1:10 to 10:1
preferably from 1:6 to 4:1
more preferably from 1:6 to 1:1
e.g. 1:4

Likewise it is preferred for PL or its precursor to be provided in a non-phosphorylated form, to avoid situations where the dephosphorylation step may be compromised. It is pointed out that only PL is capable of passing from the plasma through the cellular membranes into most cells where it is subsequently converted into pyridoxal phosphate (PLP), the active intracellular form of PLP. Also as will be explained elsewhere herein, PL itself plays a very active role in certain physiologically important reactions relevant to the present invention. For these reasons PL itself is a preferred form of vitamin B6 in the context of the present invention.

Vitamin B 12 may be used in the form of cyanocobalamin or hydroxycabalamin or both.

"Intrinsic factor" in this art, in the context of vitamin B12 denotes substances (which are for example in nature released by the gastric mucosa of the stomach when functioning normally) with which vitamin B12 forms complexes to facilitate absorption.

Advantageously the vitamin B6 is galenically formulated to be released over a period of 2 to 8 hours, whereas the vitamin B12 (with or without intrinsic factor) is formulated to be released in less than ½ hour. More particularly the vitamin B6 is galenically formulated to be released over a period of 2 to 8 hours, preferably 3 to 6 hours, more preferably 4 to 6 hours and the B12 over a period of 5 - 30 minutes.

Preferably, the folate as well is galenically formulated to be released by the composition in not less than 2 hours, preferably 2 to 8 hours, more preferably 3 to 6 hours, e.g. 4 to 6 hours.

The preferred compositions contain vitamin B6 and, preferably folate in a part of the composition adapted as a slow, timed release composition and containing the vitamin B12 (with or without intrinsic factor) in another part adapted for fast release. Examples of such compositions include the following:

a) a bi-layered tablet,
b) a coated tablet, containing the vitamin B12 in a rapidly dissolving coating; or
c) a pharmaceutical composition in granular form, loose or in a capsule.

Novelty and inventiveness is claimed to reside in the feature as such of combining folate and vitamin B12 in a combination, wherein the former is galenically formulated or adapted to be administered in a slow, timed release manner and the latter is formulated or adapted for fast release. This feature is considered as a further aspect of the present invention, to be applied as such or in combination with the remaining features of the invention herein disclosed.

The manner of putting that aspect of the invention into effect is as disclosed herein in conjunction with the preceding aspects of the invention.

Furthermore, apart from the proven toxicity of homocysteine, it has in addition now been found that elevated homocysteine levels in plasma are also indicative of free radical activity and of a general vitamin deficiency, and notably a deficiency of those vitamins which control free radicals in plasma. Free radicals in plasma as such, are a risk factory, which can be associated with serious diseases, notably vascular diseases. Accordingly, the invention preferably provides for the co-administration with the aforesaid

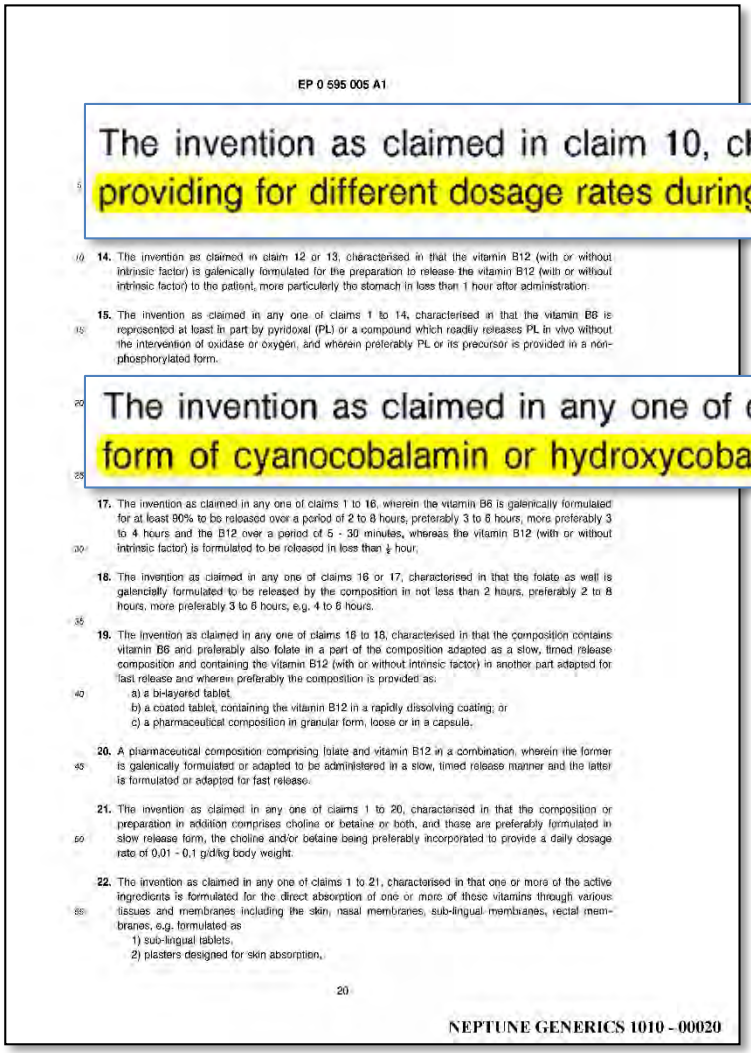
6

NEPTUNE GENERICS 1010 - 00006

Source: Ex. 1010 at 6; Paper 48 at 34.

B12 and FA Schedule

EP 005



EP 0 595 005 A1

The invention as claimed in claim 10, characterised in that the dosage regimen is time programmed, providing for different dosage rates during different periods of a course of treatment.

14. The invention as claimed in claim 12 or 13, characterised in that the vitamin B12 (with or without intrinsic factor) is galenically formulated for the preparation to release the vitamin B12 (with or without intrinsic factor) to the patient, more particularly the stomach in less than 1 hour after administration.

15. The invention as claimed in any one of claims 1 to 14, characterised in that the vitamin B6 is represented at least in part by pyridoxal (PL) or a compound which readily releases PL in vivo without the intervention of oxidase or oxygen, and wherein preferably PL or its precursor is provided in a non-phosphorylated form.

The invention as claimed in any one of claims 1 to 15, characterised in that vitamin B12 is used in the form of cyanocobalamin or hydroxycobalamin or both the the vitamin B6 being preferably represented

17. The invention as claimed in any one of claims 1 to 16, wherein the vitamin B6 is galenically formulated for at least 80% to be released over a period of 2 to 8 hours, preferably 3 to 6 hours, more preferably 3 to 4 hours and the B12 over a period of 5 - 30 minutes, whereas the vitamin B12 (with or without intrinsic factor) is formulated to be released in less than ½ hour.

18. The invention as claimed in any one of claims 16 or 17, characterised in that the folate as well is galenically formulated to be released by the composition in not less than 2 hours, preferably 2 to 8 hours, more preferably 3 to 6 hours, e.g. 4 to 6 hours.

19. The invention as claimed in any one of claims 16 to 18, characterised in that the composition contains vitamin B6 and preferably also folate in a part of the composition adapted as a slow, timed release composition and containing the vitamin B12 (with or without intrinsic factor) in another part adapted for fast release and wherein preferably the composition is provided as:
a) a bi-layered tablet,
b) a coated tablet, containing the vitamin B12 in a rapidly dissolving coating; or
c) a pharmaceutical composition in granular form, loose or in a capsule.

20. A pharmaceutical composition comprising folate and vitamin B12 in a combination, wherein the former is galenically formulated or adapted to be administered in a slow, timed release manner and the latter is formulated or adapted for fast release.

21. The invention as claimed in any one of claims 1 to 20, characterised in that the composition or preparation in addition comprises choline or betaine or both, and these are preferably formulated in slow release form, the choline and/or betaine being preferably incorporated to provide a daily dosage rate of 0,01 - 0,1 g/d/kg body weight.

22. The invention as claimed in any one of claims 1 to 21, characterised in that one or more of the active ingredients is formulated for the direct absorption of one or more of these vitamins through various tissues and membranes including the skin, nasal membranes, sub-lingual membranes, rectal membranes, e.g. formulated as
1) sub-lingual tablets,
2) plasters designed for skin absorption.

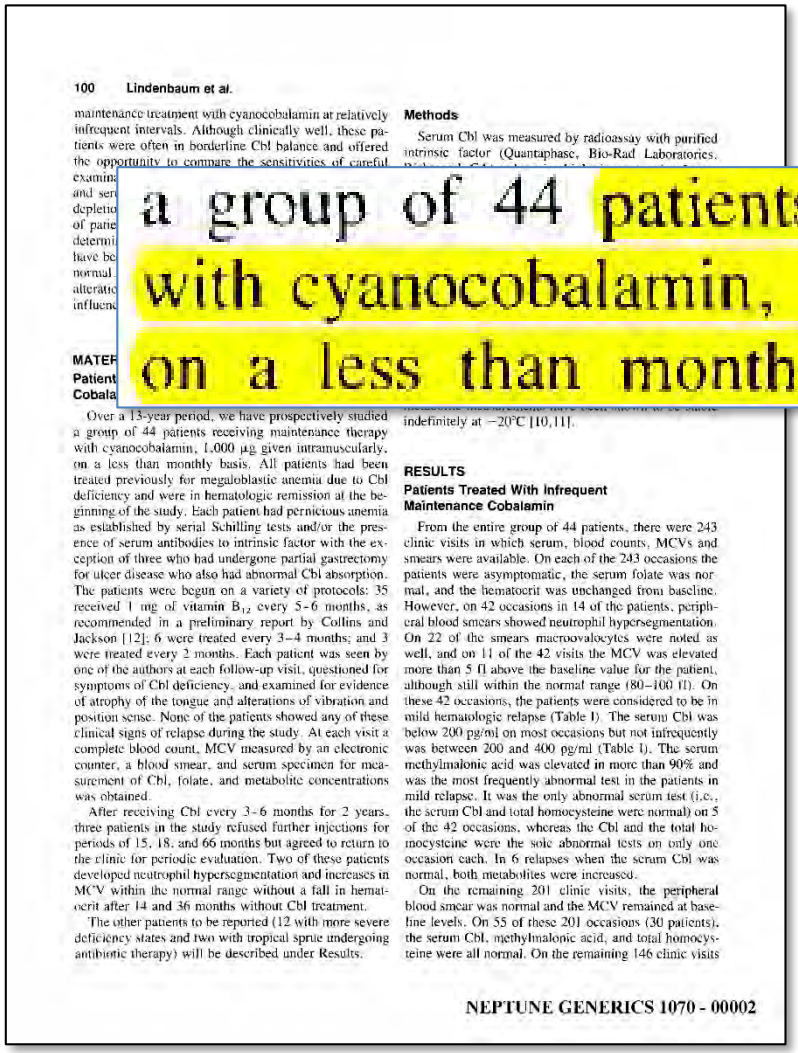
20

NEPTUNE GENERICS 1010 - 00020

Source: Ex. 1010 at claims; Paper 1 (-00240) at 22, 32, 40-43.

B12 Dose and Schedule

Lindenbaum 1990



100 Lindenbaum et al.

maintenance treatment with cyanocobalamin at relatively infrequent intervals. Although clinically well, these patients were often in borderline Cbl balance and offered the opportunity to compare the sensitivities of careful

Methods

Serum Cbl was measured by radioassay with purified intrinsic factor (Quantaphase, Bio-Rad Laboratories,

a group of 44 patients receiving maintenance therapy with cyanocobalamin, 1,000 µg given intramuscularly, on a less than monthly basis. All patients had been

MATER
Patient
Cobala

Over a 13-year period, we have prospectively studied a group of 44 patients receiving maintenance therapy with cyanocobalamin, 1,000 µg given intramuscularly, on a less than monthly basis. All patients had been treated previously for megaloblastic anemia due to Cbl deficiency and were in hematologic remission at the beginning of the study. Each patient had pernicious anemia as established by serial Schilling tests and/or the presence of serum antibodies to intrinsic factor with the exception of three who had undergone partial gastrectomy for ulcer disease who also had abnormal Cbl absorption. The patients were begun on a variety of protocols: 35 received 1 mg of vitamin B_{12} every 5–6 months, as recommended in a preliminary report by Collins and Jackson [12]; 6 were treated every 3–4 months; and 3 were treated every 2 months. Each patient was seen by one of the authors at each follow-up visit, questioned for symptoms of Cbl deficiency, and examined for evidence of atrophy of the tongue and alterations of vibration and position sense. None of the patients showed any of these clinical signs of relapse during the study. At each visit a complete blood count, MCV measured by an electronic counter, a blood smear, and serum specimen for measurement of Cbl, folate, and metabolite concentrations was obtained.

After receiving Cbl every 3–6 months for 2 years, three patients in the study refused further injections for periods of 15, 18, and 66 months but agreed to return to the clinic for periodic evaluation. Two of these patients developed neutrophil hypersegmentation and increases in MCV within the normal range without a fall in hematocrit after 14 and 36 months without Cbl treatment.

The other patients to be reported (12 with more severe deficiency states and two with tropical sprue undergoing antibiotic therapy) will be described under Results.

indefinitely at −20°C [10,11].

RESULTS

Patients Treated With Infrequent Maintenance Cobalamin

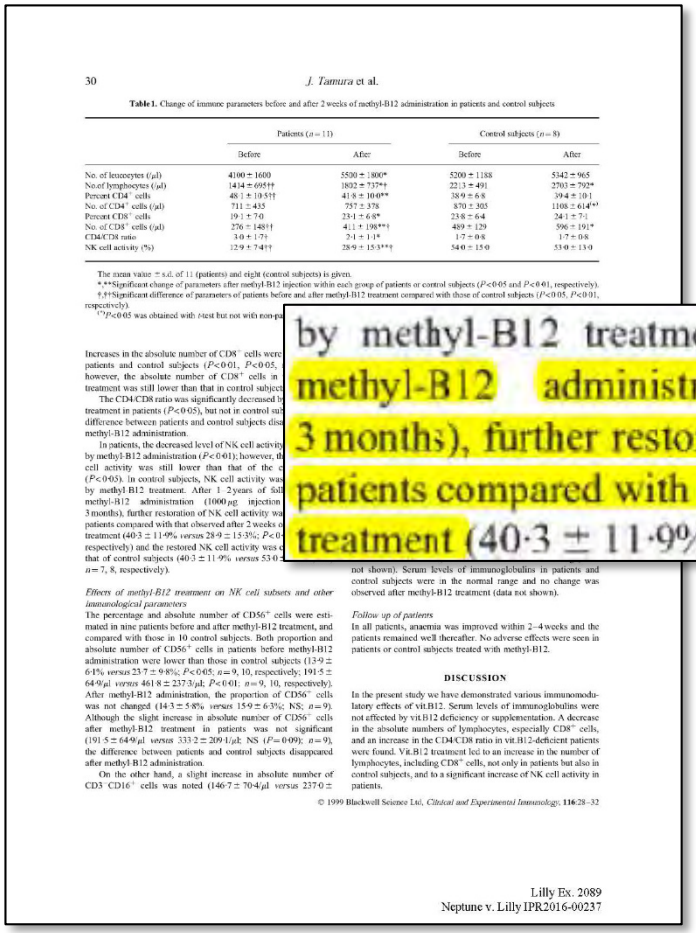
From the entire group of 44 patients, there were 243 clinic visits in which serum, blood counts, MCVs and smears were available. On each of the 243 occasions the patients were asymptomatic, the serum folate was normal, and the hematocrit was unchanged from baseline. However, on 42 occasions in 14 of the patients, peripheral blood smears showed neutrophil hypersegmentation. On 22 of the smears macroovalocytes were noted as well, and on 11 of the 42 visits the MCV was elevated more than 5 fl above the baseline value for the patient, although still within the normal range (80–100 fl). On these 42 occasions, the patients were considered to be in mild hematologic relapse (Table I). The serum Cbl was below 200 pg/ml on most occasions but not infrequently was between 200 and 400 pg/ml (Table I). The serum methylmalonic acid was elevated in more than 90% and was the most frequently abnormal test in the patients in mild relapse. It was the only abnormal serum test (i.e., the serum Cbl and total homocysteine were normal) on 5 of the 42 occasions, whereas the Cbl and the total homocysteine were the sole abnormal tests on only one occasion each. In 6 relapses when the serum Cbl was normal, both metabolites were increased.

On the remaining 201 clinic visits, the peripheral blood smear was normal and the MCV remained at baseline levels. On 55 of these 201 occasions (30 patients), the serum Cbl, methylmalonic acid, and total homocysteine were all normal. On the remaining 146 clinic visits

NEPTUNE GENERICS 1070 - 00002

B12 Dose and Schedule

Tamura



30 J. Tamura et al.

Table 1. Change of immune parameters before and after 2 weeks of methyl-B12 administration in patients and control subjects

	Patients ($n=11$)		Control subjects ($n=8$)	
	Before	After	Before	After
No. of leucocytes (/μl)	4100 ± 1600	5530 ± 1800*	5200 ± 1188	5342 ± 965
No.of lymphocytes (/μl)	1414 ± 695††	1802 ± 737*†	2213 ± 491	2703 ± 792*
Percent $CD4^+$ cells	48·1 ± 10·5††	41·8 ± 10·0**	38·9 ± 6·8	39·4 ± 10·1
No. of $CD4^+$ cells (/μl)	711 ± 435	757 ± 378	870 ± 305	1108 ± 614*§
Percent $CD8^+$ cells	19·1 ± 7·0	23·1 ± 6·8*	23·8 ± 6·4	24·1 ± 7·1
No. of $CD8^+$ cells (/μl)	276 ± 148††	411 ± 198**†	489 ± 129	596 ± 191*
CD4/CD8 ratio	3·0 ± 1·7†	2·1 ± 1·1*	1·7 ± 0·8	1·7 ± 0·8
NK cell activity (%)	12·9 ± 7·4††	28·9 ± 15·3**†	54·0 ± 15·0	53·0 ± 13·0

The mean value ± s.d. of 11 (patients) and eight (control subjects) is given.
*,**Significant change of parameters after methyl-B12 injection within each group of patients or control subjects ($P<0·05$ and $P<0·01$, respectively).
†,††Significant difference of parameters of patients before and after methyl-B12 treatment compared with those of control subjects ($P<0·05$, $P<0·01$, respectively).
§$P<0·05$ was obtained with t-test but not with non-pa

Increases in the absolute number of $CD8^+$ cells were
patients and control subjects ($P<0·01$, $P<0·05$,
however, the absolute number of $CD8^+$ cells in
treatment was still lower than that in control subject
The CD4/CD8 ratio was significantly decreased b
treatment in patients ($P<0·05$), but not in control su
difference between patients and control subjects disa
methyl-B12 administration.
In patients, the decreased level of NK cell activity
by methyl-B12 administration ($P<0·01$); however, th
cell activity was still lower than that of the c
($P<0·05$). In control subjects, NK cell activity was
by methyl-B12 treatment. After 1–2 years of foll
methyl-B12 administration (1000 μg injection
3 months), further restoration of NK cell activity wa
patients compared with that observed after 2 weeks o
treatment (40·3 ± 11·9% *versus* 28·9 ± 15·3%; $P<0·$
respectively) and the restored NK cell activity was
that of control subjects (40·3 ± 11·9% *versus* 53·0 ±
$n=7, 8$, respectively).

Effects of methyl-B12 treatment on NK cell subsets and other immunological parameters

The percentage and absolute number of $CD56^+$ cells were estimated in nine patients before and after methyl-B12 treatment, and compared with those in 10 control subjects. Both proportion and absolute number of $CD56^+$ cells in patients before methyl-B12 administration were lower than those in control subjects (13·9 ± 6·1% *versus* 23·7 ± 9·8%; $P<0·05$; $n=9$, 10, respectively; 191·5 ± 64·9/μl *versus* 461·8 ± 237·3/μl; $P<0·01$; $n=9$, 10, respectively). After methyl-B12 administration, the proportion of $CD56^+$ cells was not changed (14·3 ± 5·8% *versus* 15·9 ± 6·3%; NS; $n=9$). Although the slight increase in absolute number of $CD56^+$ cells after methyl-B12 treatment in patients was not significant (191·5 ± 64·9/μl *versus* 333·2 ± 209·1/μl; NS ($P=0·09$); $n=9$), the difference between patients and control subjects disappeared after methyl-B12 administration.
On the other hand, a slight increase in absolute number of $CD3^-CD16^+$ cells was noted (146·7 ± 70·4/μl *versus* 237·0 ±

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Lilly Ex. 2089
Neptune v. Lilly IPR2016-00237

by methyl-B12 treatment. After 1–2 years of follow up, with methyl-B12 administration (1000 μg injection for every 3 months), further restoration of NK cell activity was observed in patients compared with that observed after 2 weeks of methyl-B12 treatment (40·3 ± 11·9% *versus* 28·9 ± 15·3%; $P<0·01$; $n=7, 11,$

not shown). Serum levels of immunoglobulins in patients and control subjects were in the normal range and no change was observed after methyl-B12 treatment (data not shown).

Follow up of patients

In all patients, anaemia was improved within 2–4 weeks and the patients remained well thereafter. No adverse effects were seen in patients or control subjects treated with methyl-B12.

DISCUSSION

In the present study we have demonstrated various immunomodulatory effects of vit.B12. Serum levels of immunoglobulins were not affected by vit.B12 deficiency or supplementation. A decrease in the absolute numbers of lymphocytes, especially $CD8^+$ cells, and an increase in the CD4/CD8 ratio in vit.B12-deficient patients were found. Vit.B12 treatment led to an increase in the number of lymphocytes, including $CD8^+$ cells, not only in patients but also in control subjects, and to a significant increase of NK cell activity in patients.

B12 Dose and Schedule

Tamura

Vit.B12 augments CD8⁺ cells and NK cell activity 29

and June 1993 (age 36–83 years, median 65 years; six males and five females). Seven of 11 patients had pernicious anaemia (PA) and four had post-gastrectomy megaloblastic anaemia (PGMA). All patients showed low serum levels of vit.B12 (<85 pg/ml; normal range 230–820 pg/ml). Diagnosis was made based on medical history, macrocytic anaemia in peripheral blood, erythroblastosis with megaloblastic changes in bone marrow, low serum levels of vit.B12, the presence of anti-intrinsic factor antibody, antiparietal antibody and clinical responsiveness to vit.B12 therapy.

Thirteen haematologically and immunologically normal volunteers were included as a control group (age 26–92 years, median 72 years; five males, eight females). None showed low serum levels of vit.B12 or anaemia. All tests, including any sampling of blood, were performed with informed consent and with our hospital ethical committee's approval.

Study design

Leucocyte and lymphocyte numbers, percentage and absolute numbers of $CD4^+$ cells and $CD8^+$ cells, CD4/CD8 ratio and NK cell activity were evaluated in all patients at diagnosis and compared with the values in control subjects.

In order to examine the immunomodulatory effect of vit.B12, methylcobalamin was administrated to all patients and to eight of 13 volunteers as follows. Methyl-vit.B12 (500 μg/day; methyl-B12; mecobalamin; Eisai, Tokyo, Japan) was injected intramuscularly every other day for 2 weeks and immunophenotyping of peripheral lymphocytes and NK cell activity were evaluated as

showed high serum levels of vit.B12 (>3000 pg/ml). After 2 weeks treatment, patients were treated with vit.B12 1000 μg every 3 months as out-patients; all of them were quite well and anaemia had improved. After 1–2 years of follow up, NK cell activity was

using CD3 (Leu-4–PE) ×CD57 (Leu-7–FITC), CD3 (Leu-4–PE) ×CD16 (Leu-11a–FITC), CD57 (Leu-7–FITC) ×CD16 (Leu-11c–PE) (Becton Dickinson, Mountain View, CA), were done in eight of 11 patients and 10 of 13 control subjects for evaluation of NK cell subsets. All phenotyping was performed using a FACScan (Becton Dickinson).

NK cell activity and other tests for immune response

NK cell activity was estimated in all cases and all control subjects before and after 2 weeks of methyl-B12 treatment by the standard ^{51}Cr-release assay using K562 cells as target cells (effector:target ratio was 20:1) and results were expressed as percentage cell lysis.

In five patients, phytohaemagglutinin (PHA)-, Con A- and PWM-stimulated lymphocyte blast formation were measured, and antibody-dependent cell-mediated cytotoxicity (ADCC) was also evaluated in four patients. In all patients and control subjects serum levels of IgG, IgA and IgM were evaluated before and after methyl-B12 administration.

Statistical analysis

Statistical analysis were conducted with paired *t*-test for the comparison within each group of patients and control subjects and with unpaired *t*-test for the comparison between the two groups as indicated in the legend for Table 1. Obtained *P* values were re-estimated with Wilcoxon signed rank test and Mann–Whitney rank sum test. Significance was defined as follows: both *t*-test and non-parametric method showed $P<0{\cdot}05$. *P* values <0·05 obtained with *t*-test but not with non-parametric analysis were regarded as not significant but showing a tendency. Analysed values were represented as mean ± s.d.

RESULTS

Immunophenotyping and NK cell activity in patients and control subjects before methyl-B12 administration

Although no significant difference in leucocyte counts was noted between patients ($n=11$) and control subjects ($n=13$) (4100 ± 1600/μl *versus* 5363 ± 1367/μl; NS), the lymphocyte counts were significantly decreased in patients compared with control subjects (1414 ± 695/μl *versus* 2110 ± 669/μl; $P<0{\cdot}01$).

The proportion of $CD4^+$ cells was also significantly elevated in

and after methyl-B12 administration in both patients and control subjects are summarized in Table 1. The leucocyte counts and lymphocyte counts of patients were increased significantly after methyl-B12 treatment ($P<0{\cdot}05$). After treatment, the lymphocyte counts was still significantly lower in patients than in control subjects ($P<0{\cdot}05$). Interestingly, an increase in the lymphocyte counts was observed even in control subjects ($P<0{\cdot}05$).

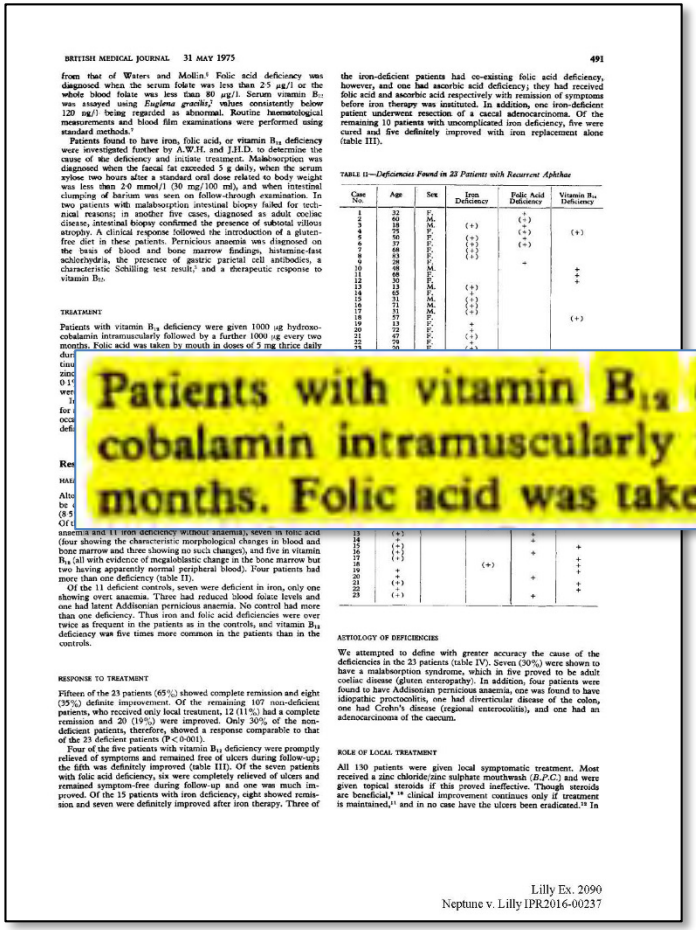
As shown in Table 1, a significant decrease of percentage $CD4^+$ cells was observed in patients after treatment ($P<0{\cdot}01$), while no significant change was noted in control subjects. No significant change of the absolute number of $CD4^+$ cells was observed in patients after methyl-B12 treatment, but a slight increase was observed in control subjects (NS but tendency).

An increase in percentage $CD8^+$ cells after methyl-B12 treatment was noted in patients ($P<0{\cdot}05$), but not in control subjects.

Source: Ex. 2089 at 29; Paper 48 at 30.

B12 Dose and Schedule

Wray



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from that of Waters and Mollin.[1] Folic acid deficiency was diagnosed when the serum folate was less than 2·5 µg/l or the whole blood folate was less than 80 µg/l. Serum vitamin B_{12} was assayed using *Euglena gracilis*;[2] values consistently below 120 ng/l being regarded as abnormal. Routine haematological measurements and blood film examinations were performed using standard methods.[3]

Patients found to have iron, folic acid, or vitamin B_{12} deficiency were investigated further by A.W.H. and J.H.D. to determine the cause of the deficiency and initiate treatment. Malabsorption was diagnosed when the faecal fat exceeded 5 g daily, when the serum xylose two hours after a standard oral dose related to body weight was less than 2·0 mmol/l (30 mg/100 ml), and when intestinal clumping of barium was seen on follow-through examination. In two patients with malabsorption intestinal biopsy failed for technical reasons; in another five cases, diagnosed as adult coeliac disease, intestinal biopsy confirmed the presence of subtotal villous atrophy. A clinical response followed the introduction of a gluten-free diet in these patients. Pernicious anaemia was diagnosed on the basis of blood and bone marrow findings, histamine-fast achlorhydria, the presence of gastric parietal cell antibodies, a characteristic Schilling test result,[3] and a therapeutic response to vitamin B_{12}.

TREATMENT

Patients with vitamin B_{12} deficiency were given 1000 µg hydroxocobalamin intramuscularly followed by a further 1000 µg every two months. Folic acid was taken by mouth in doses of 5 mg thrice daily

the iron-deficient patients had co-existing folic acid deficiency, however, and one had ascorbic acid deficiency; they had received folic acid and ascorbic acid respectively with remission of symptoms before iron therapy was instituted. In addition, one iron-deficient patient underwent resection of a caecal adenocarcinoma. Of the remaining 10 patients with uncomplicated iron deficiency, five were cured and five definitely improved with iron replacement alone (table III).

TABLE II—*Deficiencies Found in 23 Patients with Recurrent Aphthae*

Case No.	Age	Sex	Iron Deficiency	Folic Acid Deficiency	Vitamin B_{12} Deficiency
1	32	F		(+)	
2	60	M		+	
3	38	M	(+)	(+)	(+)
4	55	F		+	
5	50	F	(+)	(+)	
6	37	F	(+)	(+)	
7	68	F	(+)		
8	63	F	(+)	+	
9	28	M			
10	68	M		+	+
11	68	F			+
12	33	F			+
13	34	M	(+)		
14	62	F		+	
15	31	M	(+)		
16	71	M	(+)		
17	31	M	(+)		
18	57	F			(+)
19	13	F	+		
20	72	F			
21	59	F	(+)		
22	29	F	+		
23	28	F	(+)		

ETIOLOGY OF DEFICIENCIES

We attempted to define with greater accuracy the cause of the deficiencies in the 23 patients (table IV). Seven (30%) were shown to have a malabsorption syndrome, which in five proved to be adult coeliac disease (gluten enteropathy). In addition, four patients were found to have Addisonian pernicious anaemia, one was found to have idiopathic proctocolitis, one had diverticular disease of the colon, one had Crohn's disease (regional enterocolitis), and one had an adenocarcinoma of the caecum.

ROLE OF LOCAL TREATMENT

All 130 patients were given local symptomatic treatment. Most received a zinc chloride/zinc sulphate mouthwash (*B.P.C.*) and were given topical steroids if this proved ineffective. Though steroids are beneficial,[9 10] clinical improvement continues only if treatment is maintained,[11] and in no case have the ulcers been eradicated.[12] In

RESPONSE TO TREATMENT

Fifteen of the 23 patients (65%) showed complete remission and eight (35%) definite improvement. Of the remaining 107 non-deficient patients, who received only local treatment, 12 (11%) had a complete remission and 20 (19%) were improved. Only 30% of the non-deficient patients, therefore, showed a response comparable to that of the 23 deficient patients ($P < 0{\cdot}001$).

Four of the five patients with vitamin B_{12} deficiency were promptly relieved of symptoms and remained free of ulcers during follow-up; the fifth was definitely improved (table III). Of the seven patients with folic acid deficiency, six were completely relieved of ulcers and remained symptom-free during follow-up and one was much improved. Of the 15 patients with iron deficiency, eight showed remission and seven were definitely improved after iron therapy. Three of

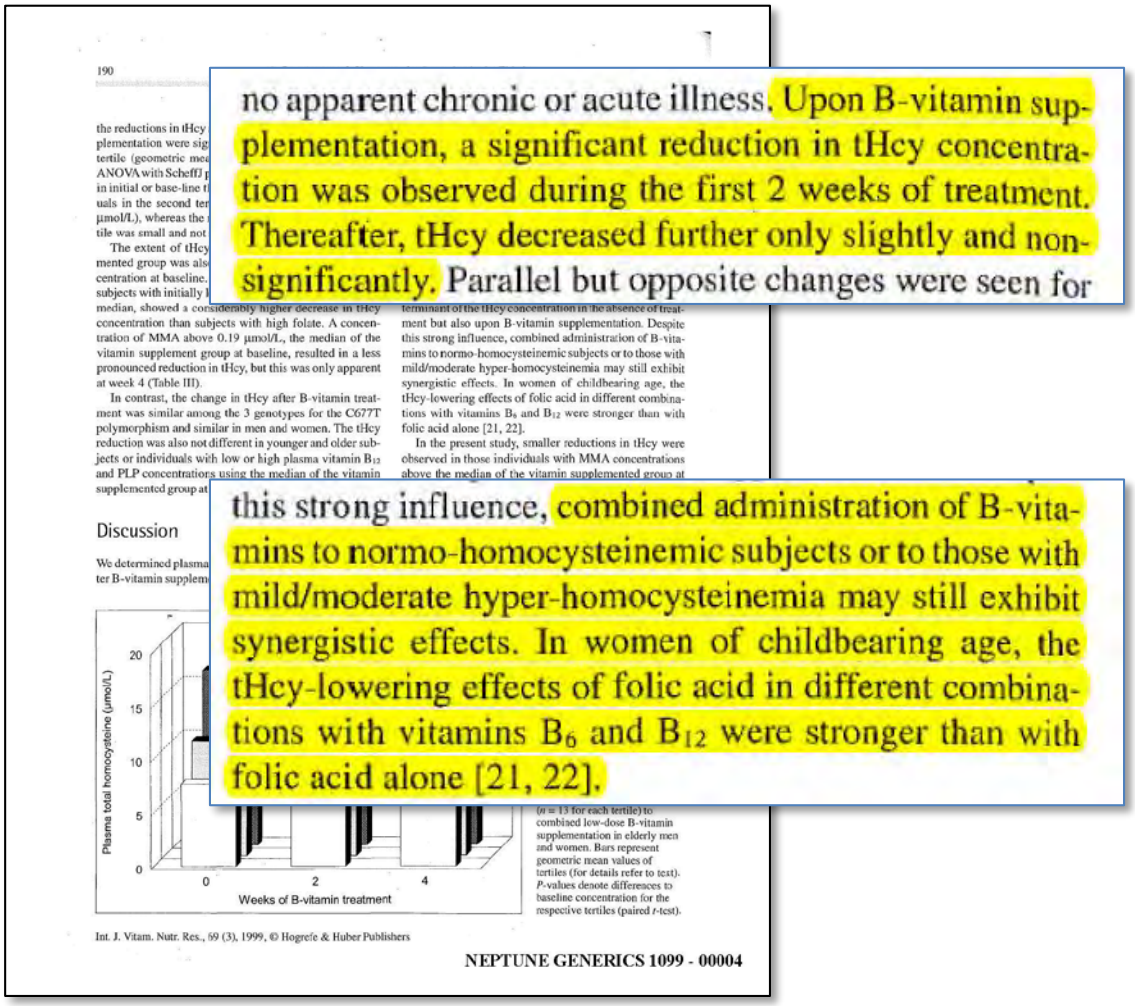
Lilly Ex. 2090
Neptune v. Lilly IPR2016-00237

Patients with vitamin B_{12} deficiency were given 1000 µg hydroxo-cobalamin intramuscularly followed by a further 1000 µg every two months. Folic acid was taken by mouth in doses of 5 mg thrice daily

Source: Ex. 2090 at 491; Paper 48 at 30.

B12 Schedule

Bronstrup II



190

the reductions in tHcy
plementation were sig
tertile (geometric mea
ANOVA with Scheffé p
in initial or base-line t
uals in the second ter
µmol/L), whereas the
tile was small and not

The extent of tHcy
mented group was als
centration at baseline.
subjects with initially
median, showed a considerably higher decrease in tHcy concentration than subjects with high folate. A concentration of MMA above 0.19 µmol/L, the median of the vitamin supplement group at baseline, resulted in a less pronounced reduction in tHcy, but this was only apparent at week 4 (Table III).

In contrast, the change in tHcy after B-vitamin treatment was similar among the 3 genotypes for the C677T polymorphism and similar in men and women. The tHcy reduction was also not different in younger and older subjects or individuals with low or high plasma vitamin B_{12} and PLP concentrations using the median of the vitamin supplemented group at

terminant of the tHcy concentration in the absence of treatment but also upon B-vitamin supplementation. Despite this strong influence, combined administration of B-vitamins to normo-homocysteinemic subjects or to those with mild/moderate hyper-homocysteinemia may still exhibit synergistic effects. In women of childbearing age, the tHcy-lowering effects of folic acid in different combinations with vitamins B_6 and B_{12} were stronger than with folic acid alone [21, 22].

In the present study, smaller reductions in tHcy were observed in those individuals with MMA concentrations above the median of the vitamin supplemented group at

Discussion

We determined plasma
ter B-vitamin supplem

(n = 13 for each tertile) to combined low-dose B-vitamin supplementation in elderly men and women. Bars represent geometric mean values of tertiles (for details refer to text). *P*-values denote differences to baseline concentration for the respective tertiles (paired *t*-test).

Int. J. Vitam. Nutr. Res., 69 (3), 1999, © Hogrefe & Huber Publishers

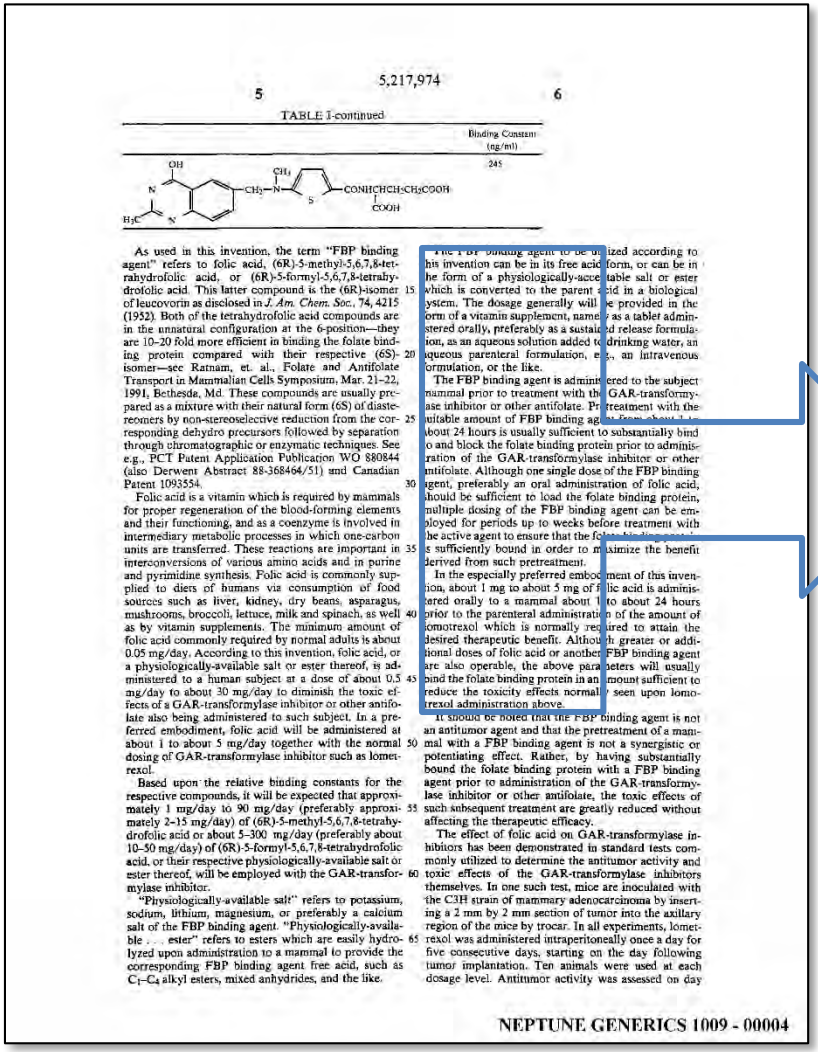
no apparent chronic or acute illness. Upon B-vitamin supplementation, a significant reduction in tHcy concentration was observed during the first 2 weeks of treatment. Thereafter, tHcy decreased further only slightly and non-significantly. Parallel but opposite changes were seen for

this strong influence, combined administration of B-vitamins to normo-homocysteinemic subjects or to those with mild/moderate hyper-homocysteinemia may still exhibit synergistic effects. In women of childbearing age, the tHcy-lowering effects of folic acid in different combinations with vitamins B_6 and B_{12} were stronger than with folic acid alone [21, 22].

Source: Ex. 1099 at 190; Paper 48 at 21.

FA Dose and Schedule

'974 Patent



5,217,974

5 6

TABLE I-continued

	Binding Constant (ng/ml)
	245

As used in this invention, the term "FBP binding agent" refers to folic acid, (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid, or (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid. This latter compound is the (6R)-isomer of leucovorin as disclosed in *J. Am. Chem. Soc.*, 74, 4215 (1952). Both of the tetrahydrofolic acid compounds are in the unnatural configuration at the 6-position—they are 10–20 fold more efficient in binding the folate binding protein compared with their respective (6S)-isomer—see Ratnam, et. al., Folate and Antifolate Transport in Mammalian Cells Symposium, Mar. 21–22, 1991, Bethesda, Md. These compounds are usually prepared as a mixture with their natural form (6S) of diastereomers by non-stereoselective reduction from the corresponding dehydro precursors followed by separation through chromatographic or enzymatic techniques See e.g., PCT Patent Application Publication WO 880844 (also Derwent Abstract 88-368464/51) and Canadian Patent 1093554.

Folic acid is a vitamin which is required by mammals for proper regeneration of the blood-forming elements and their functioning, and as a coenzyme is involved in intermediary metabolic processes in which one-carbon units are transferred. These reactions are important in interconversions of various amino acids and in purine and pyrimidine synthesis. Folic acid is commonly supplied to diets of humans via consumption of food sources such as liver, kidney, dry beans, asparagus, mushrooms, broccoli, lettuce, milk and spinach, as well as by vitamin supplements. The minimum amount of folic acid commonly required by normal adults is about 0.05 mg/day. According to this invention, folic acid, or a physiologically-available salt or ester thereof, is administered to a human subject at a dose of about 0.5 mg/day to about 30 mg/day to diminish the toxic effects of a GAR-transformylase inhibitor or other antifolate also being administered to such subject. In a preferred embodiment, folic acid will be administered at about 1 to about 5 mg/day together with the normal dosing of GAR-transformylase inhibitor such as lometrexol.

Based upon the relative binding constants for the respective compounds, it will be expected that approximately 1 mg/day to 90 mg/day (preferably approximately 2–15 mg/day) of (6R)-5-methyl-5,6,7,8-tetrahydrofolic acid or about 5–300 mg/day (preferably about 10–50 mg/day) of (6R)-5-formyl-5,6,7,8-tetrahydrofolic acid, or their respective physiologically-available salt or ester thereof, will be employed with the GAR-transformylase inhibitor.

"Physiologically-available salt" refers to potassium, sodium, lithium, magnesium, or preferably a calcium salt of the FBP binding agent. "Physiologically-available . . . ester" refers to esters which are easily hydrolyzed upon administration to a mammal to provide the corresponding FBP binding agent free acid, such as C_1–C_4 alkyl esters, mixed anhydrides, and the like.

The FBP binding agent to be utilized according to this invention can be in its free acid form, or can be in the form of a physiologically-acceptable salt or ester which is converted to the parent acid in a biological system. The dosage generally will be provided in the form of a vitamin supplement, name as a tablet administered orally, preferably as a sustained release formulation, as an aqueous solution added to drinking water, an aqueous parenteral formulation, e.g., an intravenous formulation, or the like.

The FBP binding agent is administered to the subject mammal prior to treatment with the GAR-transformylase inhibitor or other antifolate. Pretreatment with the suitable amount of FBP binding agent from about 1 to about 24 hours is usually sufficient to substantially bind to and block the folate binding protein prior to administration of the GAR-transformylase inhibitor or other antifolate. Although one single dose of the FBP binding agent, preferably an oral administration of folic acid, should be sufficient to load the folate binding protein, multiple dosing of the FBP binding agent can be employed for periods up to weeks before treatment with the active agent to ensure that the folate binding protein is sufficiently bound in order to maximize the benefit derived from such pretreatment.

In the especially preferred embodiment of this invention, about 1 mg to about 5 mg of folic acid is administered orally to a mammal about 1 to about 24 hours prior to the parenteral administration of the amount of lomotrexol which is normally required to attain the desired therapeutic benefit. Although greater or additional doses of folic acid or another FBP binding agent are also operable, the above parameters will usually bind the folate binding protein in an amount sufficient to reduce the toxicity effects normally seen upon lomotrexol administration above.

It should be noted that the FBP binding agent is not an antitumor agent and that the pretreatment of a mammal with a FBP binding agent is not a synergistic or potentiating effect. Rather, by having substantially bound the folate binding protein with a FBP binding agent prior to administration of the GAR-transformylase inhibitor or other antifolate, the toxic effects of such subsequent treatment are greatly reduced without affecting the therapeutic efficacy.

The effect of folic acid on GAR-transformylase inhibitors has been demonstrated in standard tests commonly utilized to determine the antitumor activity and toxic effects of the GAR-transformylase inhibitors themselves. In one such test, mice are inoculated with the C3H strain of mammary adenocarcinoma by inserting a 2 mm by 2 mm section of tumor into the axillary region of the mice by trocar. In all experiments, lometrexol was administered intraperitoneally once a day for five consecutive days, starting on the day following tumor implantation. Ten animals were used at each dosage level. Antitumor activity was assessed on day

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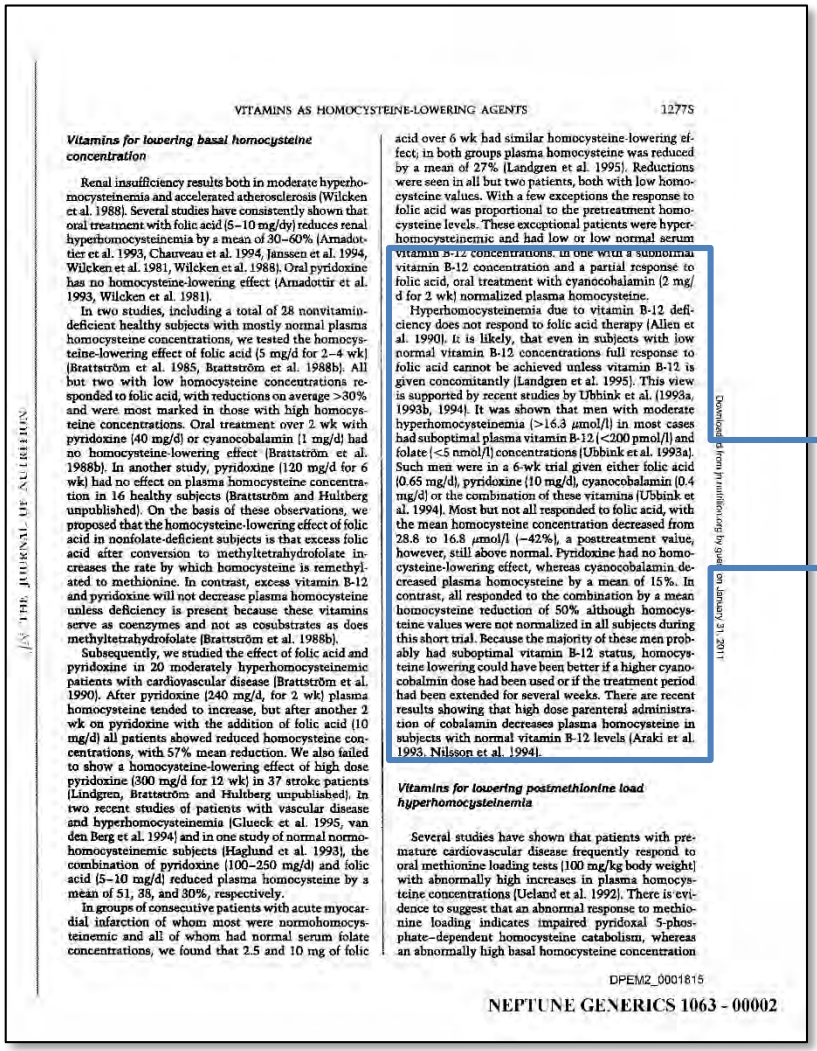
Source: Ex. 1009 at 6:22-36, 6:37-47; Paper 48 at 30.

FA and B12 Dose and Schedule

Brattström



VITAMINS AS HOMOCYSTEINE-LOWERING AGENTS 1277S

Vitamins for lowering basal homocysteine concentration

Renal insufficiency results both in moderate hyperhomocysteinemia and accelerated atherosclerosis (Wilcken et al. 1988). Several studies have consistently shown that oral treatment with folic acid (5–10 mg/day) reduces renal hyperhomocysteinemia by a mean of 30–60% (Arnadottier et al. 1993, Chauveau et al. 1994, Janssen et al. 1994, Wilcken et al. 1981, Wilcken et al. 1988). Oral pyridoxine has no homocysteine-lowering effect (Arnadottir et al. 1993, Wilcken et al. 1981).

In two studies, including a total of 28 nonvitamin-deficient healthy subjects with mostly normal plasma homocysteine concentrations, we tested the homocysteine-lowering effect of folic acid (5 mg/d for 2–4 wk) (Brattström et al. 1985, Brattström et al. 1988b). All but two with low homocysteine concentrations responded to folic acid, with reductions on average >30% and were most marked in those with high homocysteine concentrations. Oral treatment over 2 wk with pyridoxine (40 mg/d) or cyanocobalamin (1 mg/d) had no homocysteine-lowering effect (Brattström et al. 1988b). In another study, pyridoxine (120 mg/d for 6 wk) had no effect on plasma homocysteine concentration in 16 healthy subjects (Brattström and Hultberg unpublished). On the basis of these observations, we proposed that the homocysteine-lowering effect of folic acid in nonfolate-deficient subjects is that excess folic acid after conversion to methyltetrahydrofolate increases the rate by which homocysteine is remethylated to methionine. In contrast, excess vitamin B-12 and pyridoxine will not decrease plasma homocysteine unless deficiency is present because these vitamins serve as coenzymes and not as cosubstrates as does methyltetrahydrofolate (Brattström et al. 1988b).

Subsequently, we studied the effect of folic acid and pyridoxine in 20 moderately hyperhomocysteinemic patients with cardiovascular disease (Brattström et al. 1990). After pyridoxine (240 mg/d, for 2 wk) plasma homocysteine tended to increase, but after another 2 wk on pyridoxine with the addition of folic acid (10 mg/d) all patients showed reduced homocysteine concentrations, with 57% mean reduction. We also failed to show a homocysteine-lowering effect of high dose pyridoxine (300 mg/d for 12 wk) in 37 stroke patients (Lindgren, Brattström and Hultberg unpublished). In two recent studies of patients with vascular disease and hyperhomocysteinemia (Glueck et al. 1995, van den Berg et al. 1994) and in one study of normal normohomocysteinemic subjects (Haglund et al. 1993), the combination of pyridoxine (100–250 mg/d) and folic acid (5–10 mg/d) reduced plasma homocysteine by a mean of 51, 38, and 30%, respectively.

In groups of consecutive patients with acute myocardial infarction of whom most were normohomocysteinemic and all of whom had normal serum folate concentrations, we found that 2.5 and 10 mg of folic acid over 6 wk had similar homocysteine-lowering effect; in both groups plasma homocysteine was reduced by a mean of 27% (Landgren et al. 1995). Reductions were seen in all but two patients, both with low homocysteine values. With a few exceptions the response to folic acid was proportional to the pretreatment homocysteine levels. These exceptional patients were hyperhomocysteinemic and had low or low normal serum vitamin B-12 concentrations. In one with a suboptimal vitamin B-12 concentration and a partial response to folic acid, oral treatment with cyanocobalamin (2 mg/d for 2 wk) normalized plasma homocysteine.

Hyperhomocysteinemia due to vitamin B-12 deficiency does not respond to folic acid therapy (Allen et al. 1990). It is likely, that even in subjects with low normal vitamin B-12 concentrations full response to folic acid cannot be achieved unless vitamin B-12 is given concomitantly (Landgren et al. 1995). This view is supported by recent studies by Ubbink et al. (1993a, 1993b, 1994). It was shown that men with moderate hyperhomocysteinemia (>16.3 μmol/l) in most cases had suboptimal plasma vitamin B-12 (<200 pmol/l) and folate (<5 nmol/l) concentrations (Ubbink et al. 1993a). Such men were in a 6-wk trial given either folic acid (0.65 mg/d), pyridoxine (10 mg/d), cyanocobalamin (0.4 mg/d) or the combination of these vitamins (Ubbink et al. 1994). Most but not all responded to folic acid, with the mean homocysteine concentration decreased from 28.8 to 16.8 μmol/l (−42%), a posttreatment value, however, still above normal. Pyridoxine had no homocysteine-lowering effect, whereas cyanocobalamin decreased plasma homocysteine by a mean of 15%. In contrast, all responded to the combination by a mean homocysteine reduction of 50% although homocysteine values were not normalized in all subjects during this short trial. Because the majority of these men probably had suboptimal vitamin B-12 status, homocysteine lowering could have been better if a higher cyanocobalmin dose had been used or if the treatment period had been extended for several weeks. There are recent results showing that high dose parenteral administration of cobalamin decreases plasma homocysteine in subjects with normal vitamin B-12 levels (Araki et al. 1993, Nilsson et al. 1994).

Vitamins for lowering postmethionine load hyperhomocysteinemia

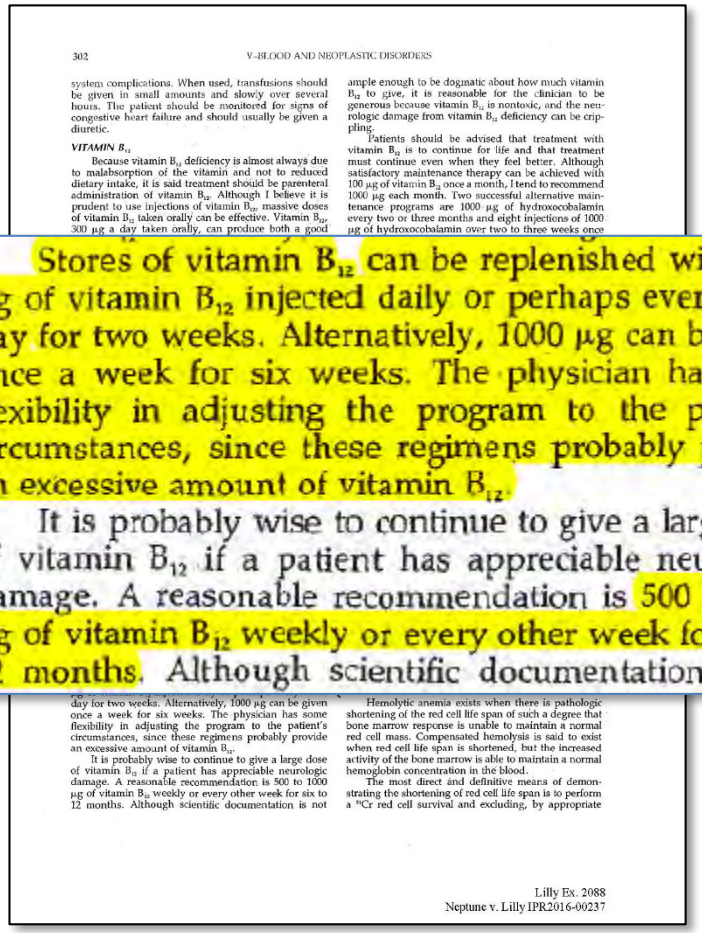
Several studies have shown that patients with premature cardiovascular disease frequently respond to oral methionine loading tests (100 mg/kg body weight) with abnormally high increases in plasma homocysteine concentrations (Ueland et al. 1992). There is evidence to suggest that an abnormal response to methionine loading indicates impaired pyridoxal 5-phosphate–dependent homocysteine catabolism, whereas an abnormally high basal homocysteine concentration

Hyperhomocysteinemia due to vitamin B-12 deficiency does not respond to folic acid therapy (Allen et al. 1990). It is likely, that even in subjects with low normal vitamin B-12 concentrations full response to folic acid cannot be achieved unless vitamin B-12 is given concomitantly (Landgren et al. 1995). This view is supported by recent studies by Ubbink et al. (1993a, 1993b, 1994). It was shown that men with moderate hyperhomocysteinemia (>16.3 μmol/l) in most cases had suboptimal plasma vitamin B-12 (<200 pmol/l) and folate (<5 nmol/l) concentrations (Ubbink et al. 1993a). Such men were in a 6-wk trial given either folic acid (0.65 mg/d), pyridoxine (10 mg/d), cyanocobalamin (0.4 mg/d) or the combination of these vitamins (Ubbink et al. 1994). Most but not all responded to folic acid, with the mean homocysteine concentration decreased from 28.8 to 16.8 μmol/l (−42%), a posttreatment value, however, still above normal. Pyridoxine had no homocysteine-lowering effect, whereas cyanocobalamin decreased plasma homocysteine by a mean of 15%. In contrast, all responded to the combination by a mean homocysteine reduction of 50% although homocysteine values were not normalized in all subjects during this short trial. Because the majority of these men probably had suboptimal vitamin B-12 status, homocysteine lowering could have been better if a higher cyanocobalmin dose had been used or if the treatment period had been extended for several weeks. There are recent results showing that high dose parenteral administration of cobalamin decreases plasma homocysteine in subjects with normal vitamin B-12 levels (Araki et al. 1993, Nilsson et al. 1994).

Source: Ex. 1063 at 1277S; Paper 48 at 21.

FA and B12 Dose and Schedule

Beutler



302 V–BLOOD AND NEOPLASTIC DISORDERS

system complications. When used, transfusions should be given in small amounts and slowly over several hours. The patient should be monitored for signs of congestive heart failure and should usually be given a diuretic.

VITAMIN B_{12}

Because vitamin B_{12} deficiency is almost always due to malabsorption of the vitamin and not to reduced dietary intake, it is said treatment should be parenteral administration of vitamin B_{12}. Although I believe it is prudent to use injections of vitamin B_{12}, massive doses of vitamin B_{12} taken orally can be effective. Vitamin B_{12}, 300 μg a day taken orally, can produce both a good

ample enough to be dogmatic about how much vitamin B_{12} to give, it is reasonable for the clinician to be generous because vitamin B_{12} is nontoxic, and the neurologic damage from vitamin B_{12} deficiency can be crippling.

Patients should be advised that treatment with vitamin B_{12} is to continue for life and that treatment must continue even when they feel better. Although satisfactory maintenance therapy can be achieved with 100 μg of vitamin B_{12} once a month, I tend to recommend 1000 μg each month. Two successful alternative maintenance programs are 1000 μg of hydroxocobalamin every two or three months and eight injections of 1000 μg of hydroxocobalamin over two to three weeks once

Stores of vitamin B_{12} can be replenished with 1000 μg of vitamin B_{12} injected daily or perhaps every other day for two weeks. Alternatively, 1000 μg can be given once a week for six weeks. The physician has some flexibility in adjusting the program to the patient's circumstances, since these regimens probably provide an excessive amount of vitamin B_{12}.

It is probably wise to continue to give a large dose of vitamin B_{12} if a patient has appreciable neurologic damage. A reasonable recommendation is 500 to 1000 μg of vitamin B_{12} weekly or every other week for six to 12 months. Although scientific documentation is not

day for two weeks. Alternatively, 1000 μg can be given once a week for six weeks. The physician has some flexibility in adjusting the program to the patient's circumstances, since these regimens probably provide an excessive amount of vitamin B_{12}.

It is probably wise to continue to give a large dose of vitamin B_{12} if a patient has appreciable neurologic damage. A reasonable recommendation is 500 to 1000 μg of vitamin B_{12} weekly or every other week for six to 12 months. Although scientific documentation is not

Hemolytic anemia exists when there is pathologic shortening of the red cell life span of such a degree that bone marrow response is unable to maintain a normal red cell mass. Compensated hemolysis is said to exist when red cell life span is shortened, but the increased activity of the bone marrow is able to maintain a normal hemoglobin concentration in the blood.

The most direct and definitive means of demonstrating the shortening of red cell life span is to perform a ^{51}Cr red cell survival and excluding, by appropriate

Lilly Ex. 2088
Neptune v. Lilly IPR2016-00237

Most patients will respond to treatment of folic acid deficiency with 1 mg of folic acid a day taken orally.

Source: Ex. 2088 at 302; Paper 48 at 30.

STATE OF THE ART

State of the Art

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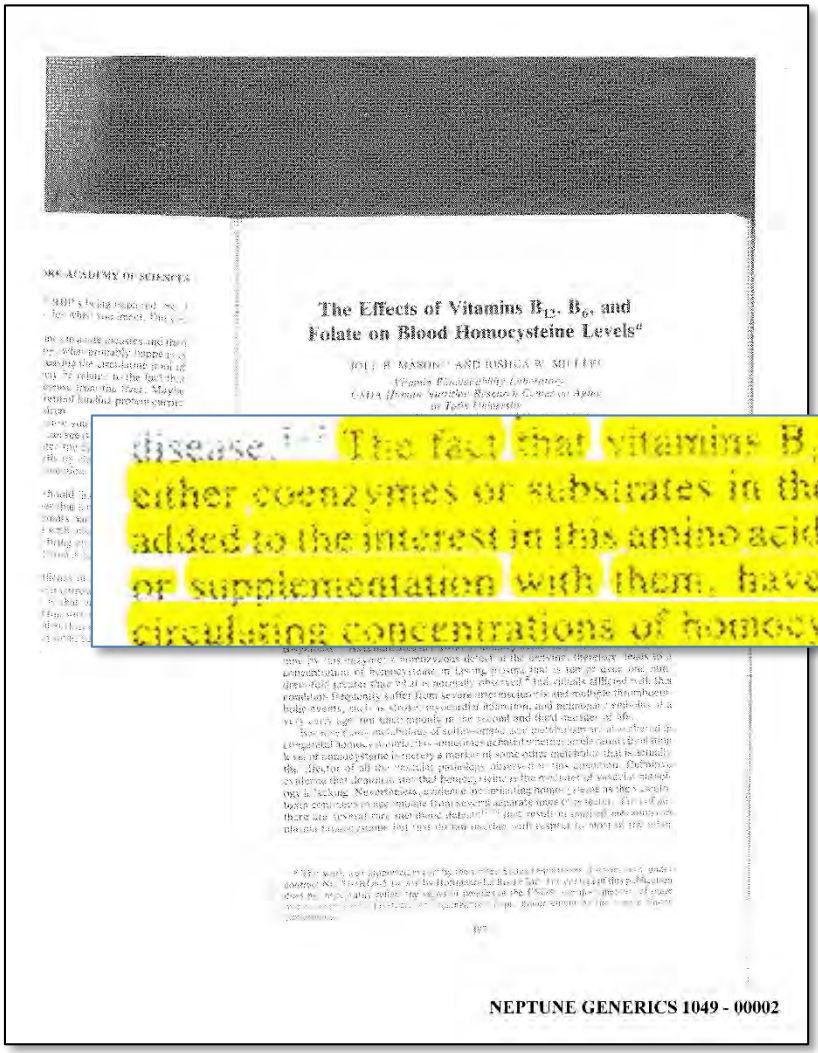


The Effects of Vitamins B_{12}, B_6, and Folate on Blood Homocysteine Levels[a]

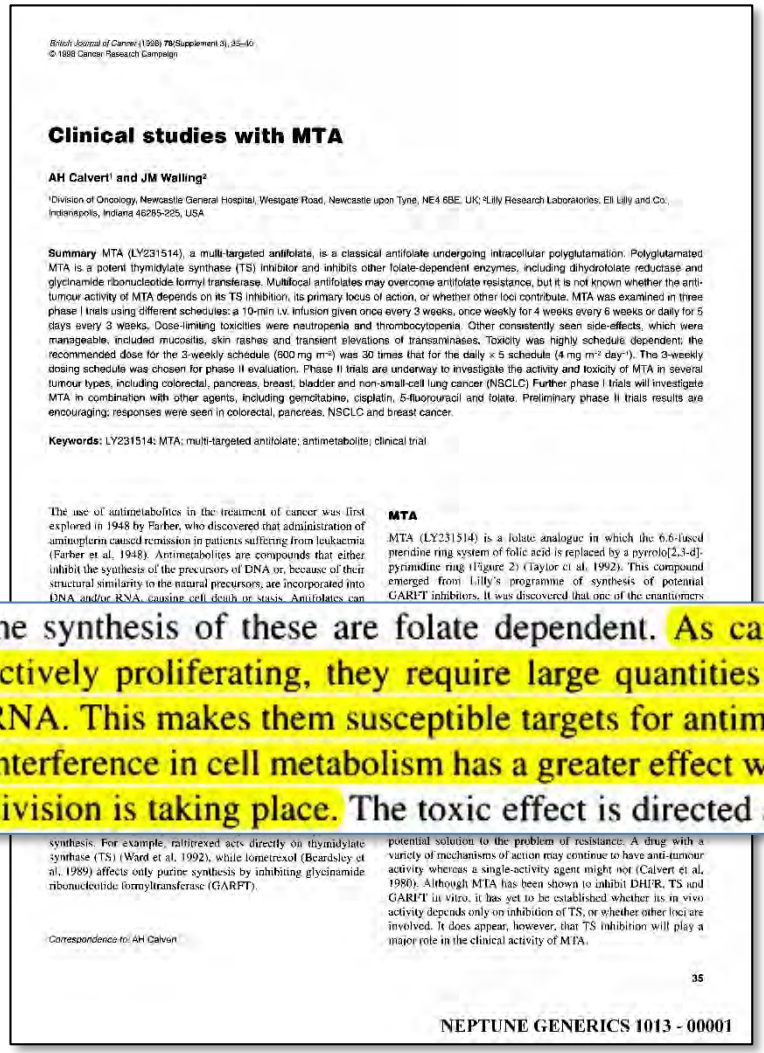
NEPTUNE GENERICS 1049 - 00002

disease.[1–7] The fact that vitamins B_{12}, B_6, and folate all play important roles as either coenzymes or substrates in the metabolism of homocysteine[8–11] has greatly added to the interest in this amino acid because deficiencies of these three vitamins or supplementation with them, have been found to have substantial impact on circulating concentrations of homocysteine in selected situations.

Source: Ex. 1049 at 197; Paper 48 at 20.

State of the Art

Calvert 1998



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Clinical studies with MTA

AH Calvert[1] and JM Walling[2]

[1]Division of Oncology, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne, NE4 6BE, UK; [2]Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Indiana 46285-225, USA

Summary MTA (LY231514), a multi-targeted antifolate, is a classical antifolate undergoing intracellular polyglutamation. Polyglutamated MTA is a potent thymidylate synthase (TS) inhibitor and inhibits other folate-dependent enzymes, including dihydrofolate reductase and glycinamide ribonucleotide formyl transferase. Multifocal antifolates may overcome antifolate resistance, but it is not known whether the anti-tumour activity of MTA depends on its TS inhibition, its primary locus of action, or whether other loci contribute. MTA was examined in three phase I trials using different schedules: a 10-min i.v. infusion given once every 3 weeks, once weekly for 4 weeks every 6 weeks or daily for 5 days every 3 weeks. Dose-limiting toxicities were neutropenia and thrombocytopenia. Other consistently seen side-effects, which were manageable, included mucositis, skin rashes and transient elevations of transaminases. Toxicity was highly schedule dependent; the recommended dose for the 3-weekly schedule (600 mg m^{-2}) was 30 times that for the daily × 5 schedule (4 mg m^{-2} day^{-1}). The 3-weekly dosing schedule was chosen for phase II evaluation. Phase II trials are underway to investigate the activity and toxicity of MTA in several tumour types, including colorectal, pancreas, breast, bladder and non-small-cell lung cancer (NSCLC) Further phase I trials will investigate MTA in combination with other agents, including gemcitabine, cisplatin, 5-fluorouracil and folate. Preliminary phase II trials results are encouraging; responses were seen in colorectal, pancreas, NSCLC and breast cancer.

Keywords: LY231514; MTA; multi-targeted antifolate; antimetabolite; clinical trial

The use of antimetabolites in the treatment of cancer was first explored in 1948 by Farber, who discovered that administration of aminopterin caused remission in patients suffering from leukaemia (Farber et al, 1948). Antimetabolites are compounds that either inhibit the synthesis of the precursors of DNA or, because of their structural similarity to the natural precursors, are incorporated into DNA and/or RNA, causing cell death or stasis. Antifolates can

MTA

MTA (LY231514) is a folate analogue in which the 6,6-fused pteridine ring system of folic acid is replaced by a pyrrolo[2,3-d]-pyrimidine ring (Figure 2) (Taylor et al, 1992). This compound emerged from Lilly's programme of synthesis of potential GARFT inhibitors. It was discovered that one of the enantiomers

the synthesis of these are folate dependent. As cancer cells are actively proliferating, they require large quantities of DNA and RNA. This makes them susceptible targets for antimetabolities, as interference in cell metabolism has a greater effect when rapid cell division is taking place. The toxic effect is directed at all prolifer-

synthesis. For example, raltitrexed acts directly on thymidylate synthase (TS) (Ward et al, 1992), while lometrexol (Beardsley et al, 1989) affects only purine synthesis by inhibiting glycinamide ribonucleotide formyltransferase (GARFT).

potential solution to the problem of resistance. A drug with a variety of mechanisms of action may continue to have anti-tumour activity whereas a single-activity agent might not (Calvert et al, 1980). Although MTA has been shown to inhibit DHFR, TS and GARFT in vitro, it has yet to be established whether its in vivo activity depends only on inhibition of TS, or whether other loci are involved. It does appear, however, that TS inhibition will play a major role in the clinical activity of MTA.

Correspondence to: AH Calvert

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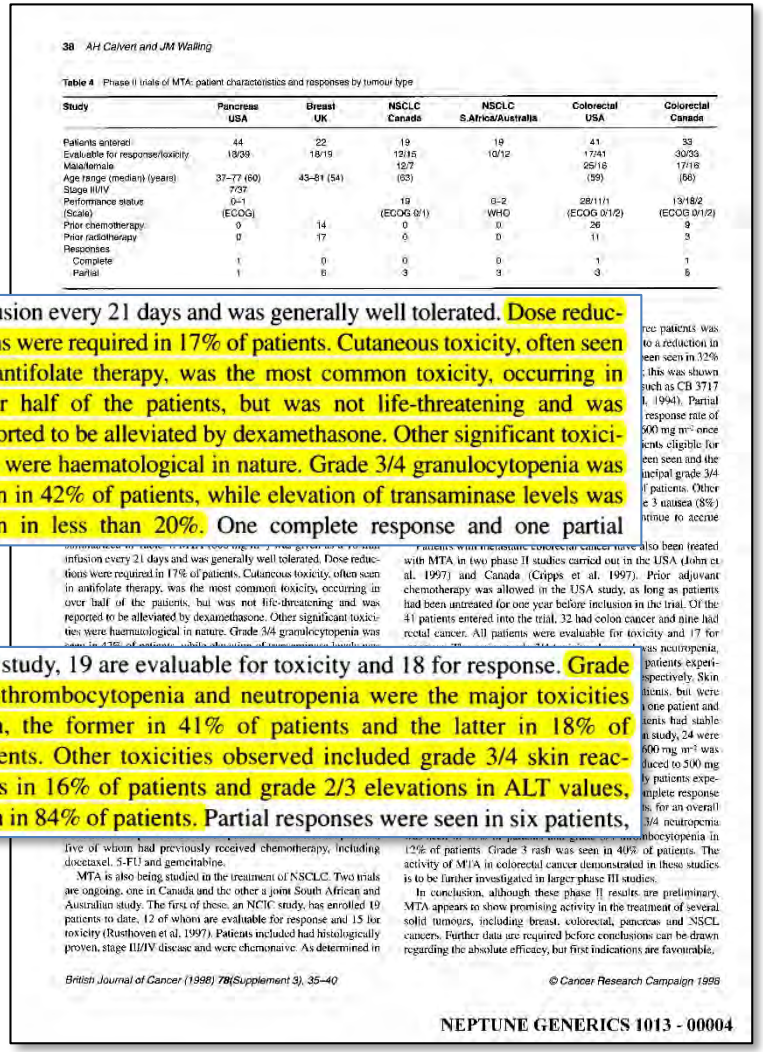

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Table 4 Phase II trials of MTA: patient characteristics and responses by tumour type

Study	Pancreas USA	Breast UK	NSCLC Canada	NSCLC S.Africa/Australia	Colorectal USA	Colorectal Canada
Patients entered	44	22	19	19	41	33
Evaluable for response/toxicity	18/39	18/19	12/15	10/12	17/41	30/33
Male/female			12/7		25/16	17/16
Age range (median) (years)	37–77 (60)	43–81 (54)	(63)		(59)	(66)
Stage III/IV	7/37					
Performance status (Scale)	0–1 (ECOG)		19 (ECOG 0/1)	0–2 WHO	28/11/1 (ECOG 0/1/2)	13/18/2 (ECOG 0/1/2)
Prior chemotherapy	0	14	0	0	26	9
Prior radiotherapy	9	17	6	0	11	3
Responses						
Complete	1	0	0	0	1	1
Partial	1	5	3	3	3	5

infusion every 21 days and was generally well tolerated. Dose reductions were required in 17% of patients. Cutaneous toxicity, often seen in antifolate therapy, was the most common toxicity, occurring in over half of the patients, but was not life-threatening and was reported to be alleviated by dexamethasone. Other significant toxicities were haematological in nature. Grade 3/4 granulocytopenia was seen in 42% of patients, while elevation of transaminase levels was seen in less than 20%. One complete response and one partial

this study, 19 are evaluable for toxicity and 18 for response. Grade 3/4 thrombocytopenia and neutropenia were the major toxicities seen, the former in 41% of patients and the latter in 18% of patients. Other toxicities observed included grade 3/4 skin reactions in 16% of patients and grade 2/3 elevations in ALT values, seen in 84% of patients. Partial responses were seen in six patients,

five of whom had previously received chemotherapy, including docetaxel, 5-FU and gemcitabine.

MTA is also being studied in the treatment of NSCLC. Two trials are ongoing, one in Canada and the other a joint South African and Australian study. The first of these, an NCIC study, has enrolled 19 patients to date, 12 of whom are evaluable for response and 15 for toxicity (Rusthoven et al, 1997). Patients included had histologically proven, stage III/IV disease and were chemonaive. As determined in

with MTA in two phase II studies carried out in the USA (John et al, 1997) and Canada (Cripps et al, 1997). Prior adjuvant chemotherapy was allowed in the USA study, as long as patients had been untreated for one year before inclusion in the trial. Of the 41 patients entered into the trial, 32 had colon cancer and nine had rectal cancer. All patients were evaluable for toxicity and 17 for

12% of patients. Grade 3 rash was seen in 40% of patients. The activity of MTA in colorectal cancer demonstrated in these studies is to be further investigated in larger phase III studies.

In conclusion, although these phase II results are preliminary, MTA appears to show promising activity in the treatment of several solid tumours, including breast, colorectal, pancreas and NSCL cancers. Further data are required before conclusions can be drawn regarding the absolute efficacy, but first indications are favourable,

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NEPTUNE GENERICS 1013 - 00004

the phase I trials, the starting dose for the first three patients was 600 mg m^{-2}, but toxicities observed at this dose led to a reduction in the dose to 500 mg m^{-2}. Grade 3/4 neutropenia has been seen in 32% of patients, along with elevated transaminase levels; this was shown to be transient, as seen in trials of other antifolates, such as CB 3717 (Calvert et al, 1986) and raltitrexed (Burris et al, 1994). Partial

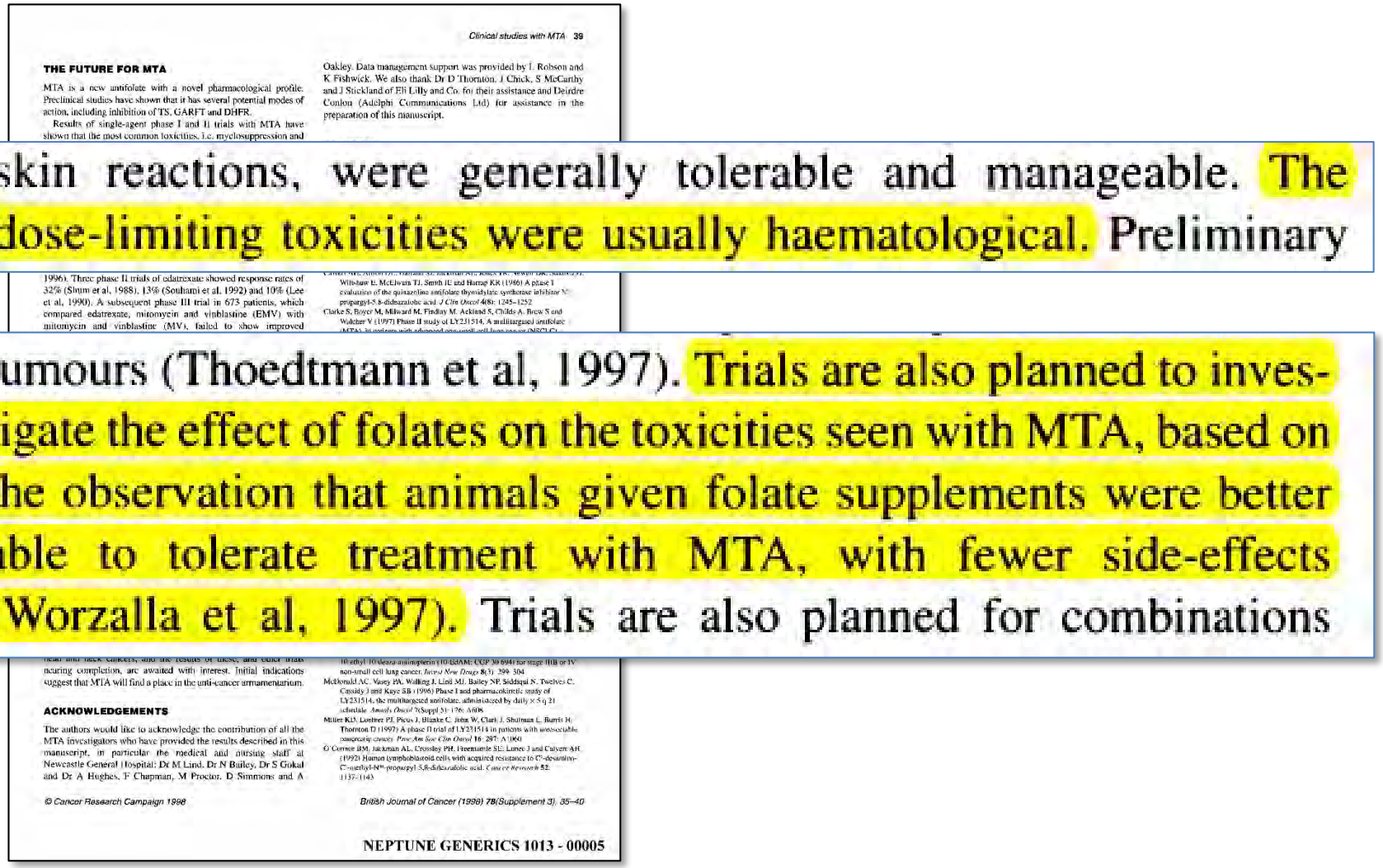
remaining seven patients had stable disease. The principal grade 3/4 toxicity was neutropenia, which occurred in 42% of patients. Other toxicities seen included grade 3/4 rash (17%), grade 3 nausea (8%) and grade 4 vomiting (8%). Both these trials continue to accrue

response. The major grade 3/4 toxicity observed was neutropenia, seen in 56% of patients, while 16% and 12% of patients experienced grade 3/4 thrombocytopenia and anaemia, respectively. Skin reactions were common, occurring in 69% of patients, but were rarely significant. A complete response was seen in one patient and

chemonaive. The recommended phase II dose of 600 mg m^{-2} was given to nine patients, but this was subsequently reduced to 500 mg m^{-2} in the remaining 24 patients, when several early patients experienced toxicities requiring dose reduction. One complete response

State of the Art

Calvert 1998



Clinical studies with MTA 39

THE FUTURE FOR MTA

MTA is a new antifolate with a novel pharmacological profile. Preclinical studies have shown that it has several potential modes of action, including inhibition of TS, GARFT and DHFR.

Results of single-agent phase I and II trials with MTA have shown that the most common toxicities, i.e. myelosuppression and

skin reactions, were generally tolerable and manageable. The dose-limiting toxicities were usually haematological. Preliminary

1996). Three phase II trials of edatrexate showed response rates of 32% (Shum et al, 1988), 13% (Souhami et al, 1992) and 10% (Lee et al, 1990). A subsequent phase III trial in 673 patients, which compared edatrexate, mitomycin and vinblastine (EMV) with mitomycin and vinblastine (MV), failed to show improved

tumours (Thoedtmann et al, 1997). Trials are also planned to investigate the effect of folates on the toxicities seen with MTA, based on the observation that animals given folate supplements were better able to tolerate treatment with MTA, with fewer side-effects (Worzalla et al, 1997). Trials are also planned for combinations

neck cancers, and the results of these, and other trials nearing completion, are awaited with interest. Initial indications suggest that MTA will find a place in the anti-cancer armamentarium.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the contribution of all the MTA investigators who have provided the results described in this manuscript, in particular the medical and nursing staff at Newcastle General Hospital: Dr M Lind, Dr N Bailey, Dr S Gokal and Dr A Hughes, F Chapman, M Proctor, D Simmons and A Oakley. Data management support was provided by I. Robson and K Fishwick. We also thank Dr D Thornton, J Chick, S McCarthy and J Strickland of Eli Lilly and Co. for their assistance and Deirdre Conlon (Adelphi Communications Ltd) for assistance in the preparation of this manuscript.

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Source: Ex. 1013 at 39; Paper 48 at 8, 11, 15, 22.

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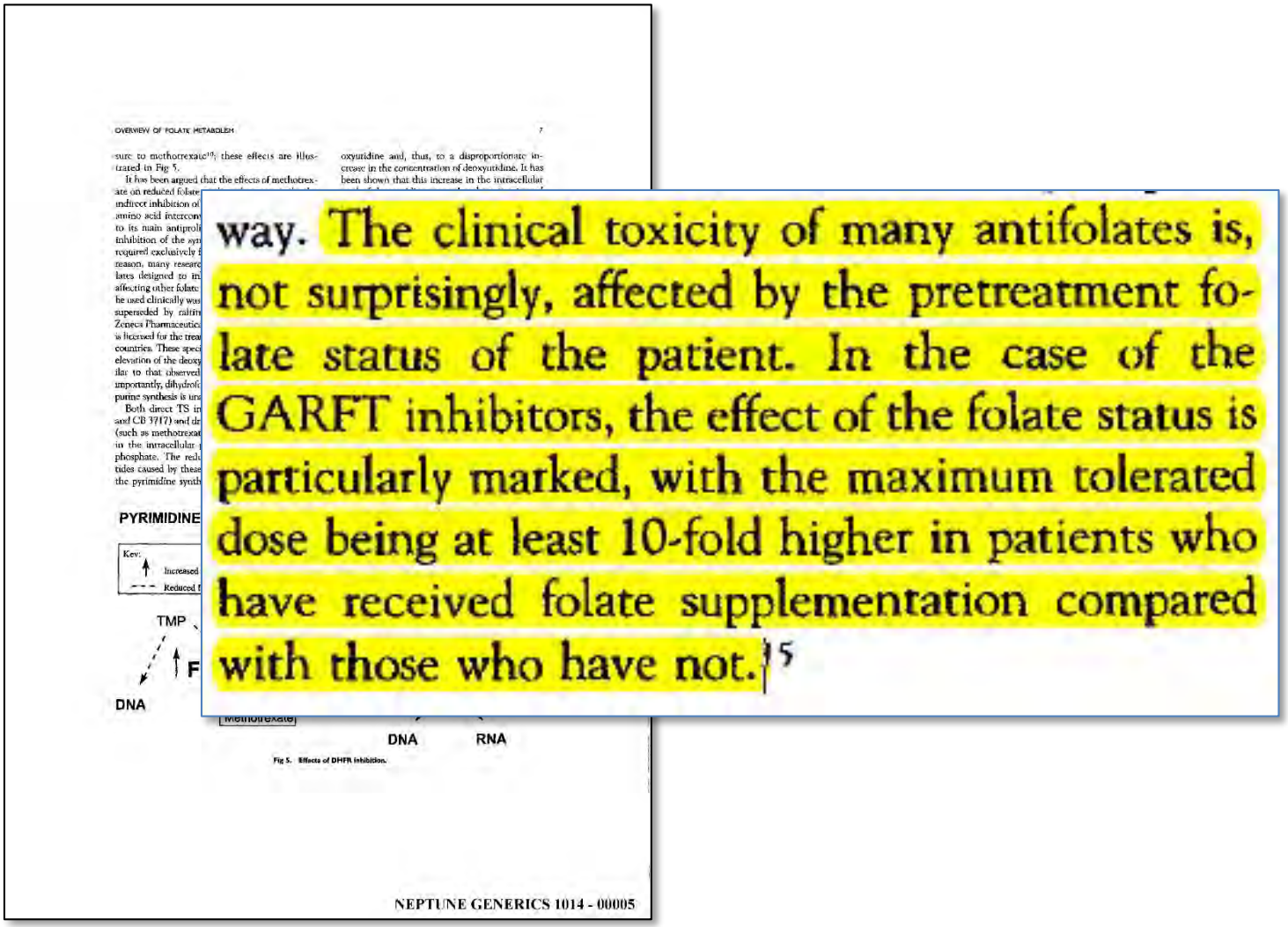

OVERVIEW OF FOLATE METABOLISM 7

sure to methotrexate[19]; these effects are illustrated in Fig 5.

It has been argued that the effects of methotrexate on reduced folate indirect inhibition of amino acid interconv to its main antiprol inhibition of the syn required exclusively f reason, many researc lates designed to in affecting other folate he used clinically was superseded by raltitr Zeneca Pharmaceutic is licensed for the treat countries. These speci elevation of the deoxy ilar to that observed importantly, dihydrofi purine synthesis is uns

Both direct TS in and CB 3717) and dr (such as methotrexat in the intracellular phosphate. The redu tides caused by these the pyrimidine synth

oxyuridine and, thus, to a disproportionate increase in the concentration of deoxyuridine. It has been shown that this increase in the intracellular

PYRIMIDINE

Key: Increased Reduced

TMP

F

DNA

Methotrexate

DNA RNA

Fig 5. Effects of DHFR inhibition.

way. The clinical toxicity of many antifolates is, not surprisingly, affected by the pretreatment folate status of the patient. In the case of the GARFT inhibitors, the effect of the folate status is particularly marked, with the maximum tolerated dose being at least 10-fold higher in patients who have received folate supplementation compared with those who have not.[15]

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Source: Ex. 1014 at 7; Paper 48 at 15, 18-19, 20, 22.

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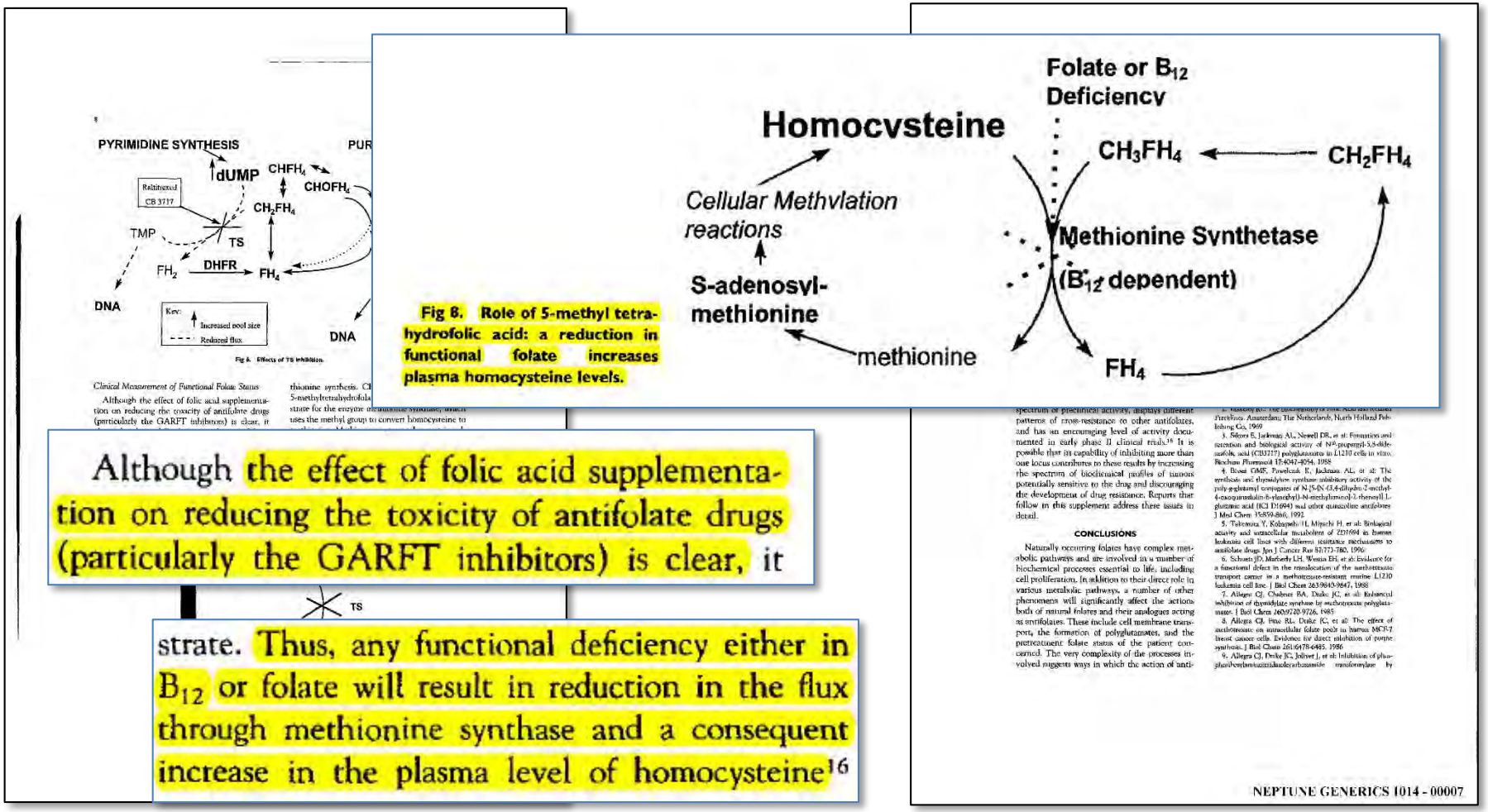

Source: Ex. 1014 at 8-9; Paper 48 at 20, 23, 25.

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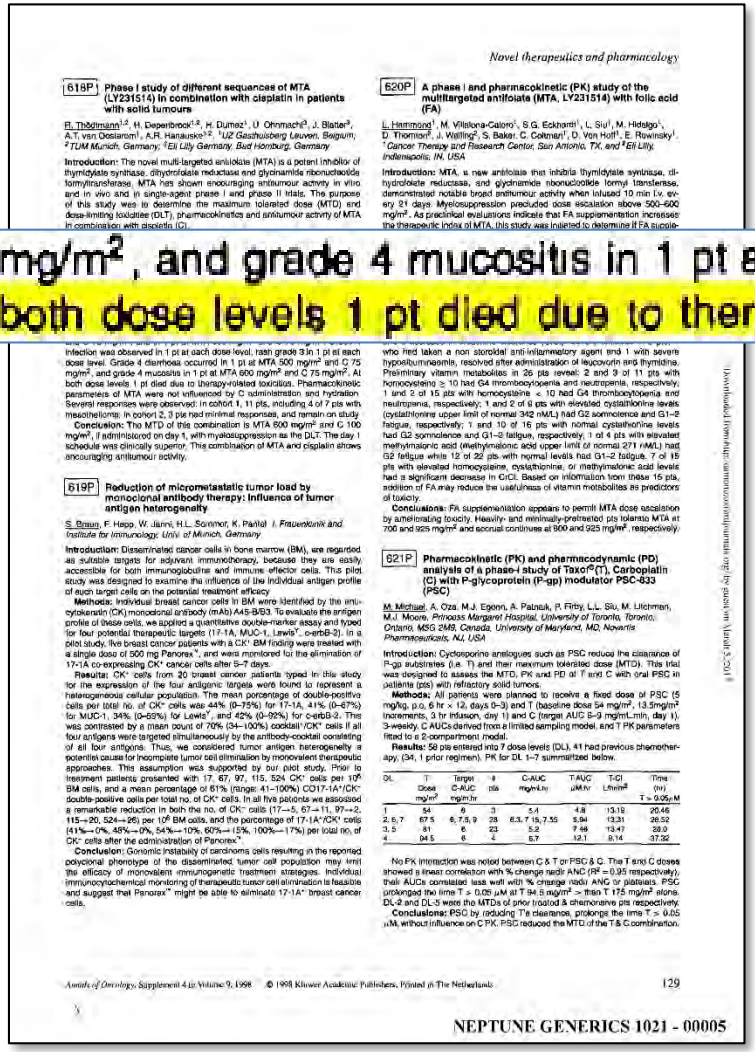

Novel therapeutics and pharmacology

618P **Phase I study of different sequences of MTA (LY231514) in combination with cisplatin in patients with solid tumours**

R. Thödtmann[1,2], H. Diepenbrock[1,2], H. Dumez[1], U. Ohnmacht[2], J. Blatter[2], A.T. van Oosterom[1], A.R. Hanauske[1,2]. [1]*UZ Gasthuisberg Leuven, Belgium;* [2]*TUM Munich, Germany;* [3]*Eli Lilly Germany, Bad Homburg, Germany*

Introduction: The novel multi-targeted antifolate (MTA) is a potent inhibitor of thymidylate synthase, dihydrofolate reductase and glycinamide ribonucleotide formyltransferase. MTA has shown encouraging antitumour activity in vitro and in vivo and in single-agent phase I and phase II trials. The purpose of this study was to determine the maximum tolerated dose (MTD) and dose-limiting toxicities (DLT), pharmacokinetics and antitumour activity of MTA in combination with cisplatin (C).

620P **A phase I and pharmacokinetic (PK) study of the multitargeted antifolate (MTA, LY231514) with folic acid (FA)**

L. Hammond[1], M. Villalona-Calero[1], S.G. Eckhardt[1], L. Siu[1], M. Hidalgo[1], D. Thomas[2], J. Watling[2], S. Baker, C. Coleman[2], D. Von Hoff[1], E. Rowinsky[1]. [1]*Cancer Therapy and Research Center, San Antonio, TX, and* [2]*Eli Lilly, Indianapolis, IN, USA*

Introduction: MTA, a new antifolate that inhibits thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyl transferase, demonstrated notable broad antitumour activity when infused 10 min i.v. every 21 days. Myelosuppression precluded dose escalation above 500–600 mg/m². As preclinical evaluations indicate that FA supplementation increases the therapeutic index of MTA, this study was initiated to determine if FA supple-

mg/m², and grade 4 mucositis in 1 pt at MTA 600 mg/m² and C 75 mg/m². At both dose levels 1 pt died due to therapy-related toxicities. Pharmacokinetic

infection was observed in 1 pt at each dose level, rash grade 3 in 1 pt at each dose level. Grade 4 diarrhoea occurred in 1 pt at MTA 500 mg/m² and C 75 mg/m², and grade 4 mucositis in 1 pt at MTA 600 mg/m² and C 75 mg/m². At both dose levels 1 pt died due to therapy-related toxicities. Pharmacokinetic parameters of MTA were not influenced by C administration and hydration. Several responses were observed: in cohort 1, 11 pts, including 4 of 7 pts with mesothelioma; in cohort 2, 3 pts had minimal responses, and remain on study.

Conclusion: The MTD of this combination is MTA 600 mg/m² and C 100 mg/m², if administered on day 1, with myelosuppression as the DLT. The day 1 schedule was clinically superior. This combination of MTA and cisplatin shows encouraging antitumour activity.

619P **Reduction of micrometastatic tumor load by monoclonal antibody therapy: influence of tumor antigen heterogeneity**

S. Braun, F. Hepp, W. Janni, H.L. Sommer, K. Pantel. *I. Frauenklinik and Institute for Immunology, Univ. of Munich, Germany*

Introduction: Disseminated cancer cells in bone marrow (BM), are regarded as suitable targets for adjuvant immunotherapy, because they are easily accessible for both immunogobulins and immune effector cells. This pilot study was designed to examine the influence of the individual antigen profile of such target cells on the potential treatment efficacy.

Methods: Individual breast cancer cells in BM were identified by the immunocytokeratin (CK) monoclonal antibody (mAb) A45-B/B3. To evaluate the antigen profile of these cells, we applied a quantitative double-marker assay and typed for four potential therapeutic targets (17-1A, MUC-1, Lewis^y, c-erbB-2). In a pilot study, five breast cancer patients with a CK$^+$ BM finding were treated with a single dose of 500 mg Panorex®, and were monitored for the elimination of 17-1A co-expressing CK$^+$ cancer cells after 5–7 days.

Results: CK$^+$ cells from 20 breast cancer patients typed in this study for the expression of the four antigenic targets were found to represent a heterogeneous cellular population. The mean percentage of double-positive cells per total no. of CK$^+$ cells was 44% (0–75%) for 17-1A, 41% (0–67%) for MUC-1, 34% (0–69%) for Lewis^y, and 42% (0–92%) for c-erbB-2. This was contrasted by a mean count of 70% (34–100%) cocktail/CK$^+$ cells if all four antigens were targeted simultaneously by the antibody-cocktail consisting of all four antigens. Thus, we considered tumor antigen heterogeneity a potential cause for incomplete tumor cell elimination by monovalent therapeutic approaches. This assumption was supported by our pilot study. Prior to treatment patients presented with 17, 67, 97, 115, 524 CK$^+$ cells per 10^6 BM cells, and a mean percentage of 61% (range: 41–100%) CO17-1A$^+$/CK$^+$ double-positive cells per total no. of CK$^+$ cells. In all five patients we assessed a remarkable reduction in both the no. of CK$^+$ cells (17→5, 67→11, 97→2, 115→20, 524→26) per 10^6 BM cells, and the percentage of (17-1A$^+$/CK$^+$ cells (41%→0%, 48%→0%, 54%→10%, 60%→15%, 100%→17%) per total no. of CK$^+$ cells after the administration of Panorex®.

Conclusion: Genomic instability of carcinoma cells resulting in the reported polyclonal phenotype of the disseminated tumor cell population may limit the efficacy of monovalent immunogenetic treatment strategies. Individual immunocytochemical monitoring of therapeutic tumor cell elimination is feasible and suggest that Panorex® might be able to eliminate 17-1A$^+$ breast cancer cells.

621P **Pharmacokinetic (PK) and pharmacodynamic (PD) analysis of a phase-I study of Taxol®(T), Carboplatin (C) with P-glycoprotein (P-gp) modulator PSC-833 (PSC)**

M. Michael, A. Oza, M.J. Egorin, A. Patnaik, P. Firby, L.L. Siu, M. Litchman, M.J. Moore. *Princess Margaret Hospital, University of Toronto, Toronto, Ontario, M5G 2M9, Canada, University of Maryland, MD, Novartis Pharmaceuticals, NJ, USA*

Introduction: Cyclosporine analogues such as PSC reduce the clearance of P-gp substrates (i.e. T) and their maximum tolerated dose (MTD). This trial was designed to assess the MTD, PK and PD of T and C with oral PSC in patients (pts) with refractory solid tumors.

Methods: All patients were planned to receive a fixed dose of PSC (5 mg/kg, p.o, 6 hr × 12, days 0–3) and T (baseline dose 54 mg/m², 13.5mg/m² increments, 3 hr infusion, day 1) and C (target AUC 5–9 mg/mL·min, day 1), 3-weekly. C AUCs derived from a limited sampling model, and T PK parameters fitted to a 2-compartment model.

Results: 58 pts entered into 7 dose levels (DL), 41 had previous chemotherapy, (34, 1 prior regimen). PK for DL 1–7 summarized below.

DL	T Dose mg/m²	Target C-AUC mg/mL·hr	# pts	C-AUC mg/mL·hr	T·AUC µM·hr	T·Cl L/hr/m²	Time (hr) T > 0.05 µM
1	54	6	3	5.4	4.8	13.19	20.46
2, 5, 7	67.5	6, 7.5, 9	28	6.3, 7.15, 7.55	5.84	13.31	26.52
3, 5	81	6	23	5.2	7.48	13.47	33.0
4	94.5	6	4	5.7	12.1	8.14	37.32

No PK interaction was noted between C & T or PSC & C. The T and C doses showed a linear correlation with % change nadir ANC (R^2 = 0.95 respectively); their AUCs correlated less well with % change nadir ANC or platelets. PSC prolonged the time T > 0.05 µM at T 94.5 mg/m² → this T 175 mg/m² alone. DL-2 and DL-3 were the MTDs of prior treated & chemonaive pts respectively.

Conclusions: PSC by reducing T's clearance, prolongs the time T > 0.05 µM, without influence on C PK. PSC reduced the MTD of the T & C combination.

Source: Ex. 1021 at 129; Paper 48 at 12.

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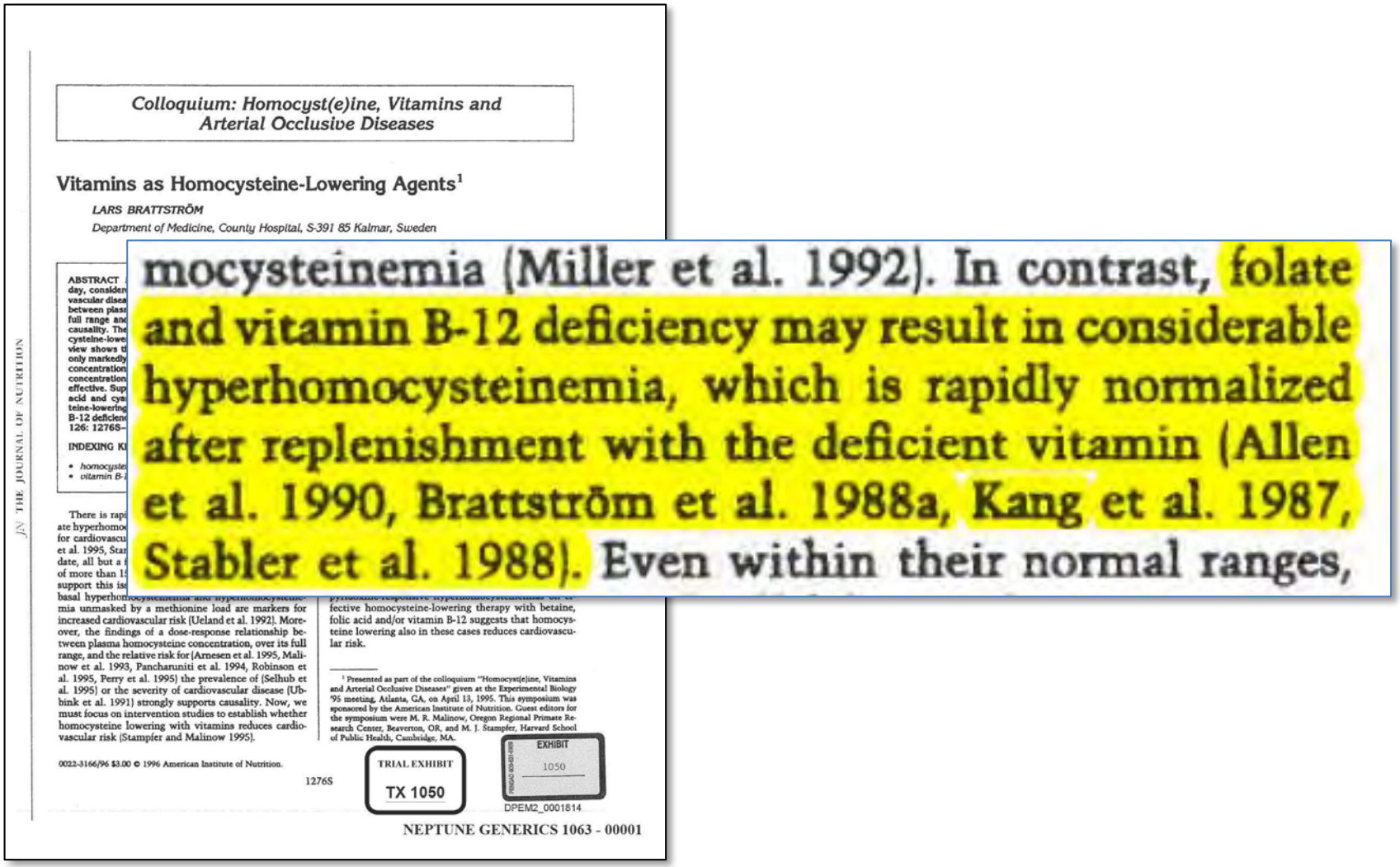

Colloquium: Homocyst(e)ine, Vitamins and Arterial Occlusive Diseases

Vitamins as Homocysteine-Lowering Agents[1]

LARS BRATTSTRÖM
Department of Medicine, County Hospital, S-391 85 Kalmar, Sweden

ABSTRACT
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basal hyperhom
mia unmasked by a methionine load are markers for increased cardiovascular risk (Ueland et al. 1992). Moreover, the findings of a dose-response relationship between plasma homocysteine concentration, over its full range, and the relative risk for (Arnesen et al. 1995, Malinow et al. 1993, Pancharuniti et al. 1994, Robinson et al. 1995, Perry et al. 1995) the prevalence of (Selhub et al. 1995) or the severity of cardiovascular disease (Ubbink et al. 1991) strongly supports causality. Now, we must focus on intervention studies to establish whether homocysteine lowering with vitamins reduces cardiovascular risk (Stampfer and Malinow 1995).

fective homocysteine-lowering therapy with betaine, folic acid and/or vitamin B-12 suggests that homocysteine lowering also in these cases reduces cardiovascular risk.

[1] Presented as part of the colloquium "Homocyst(e)ine, Vitamins and Arterial Occlusive Diseases" given at the Experimental Biology '95 meeting, Atlanta, GA, on April 13, 1995. This symposium was sponsored by the American Institute of Nutrition. Guest editors for the symposium were M. R. Malinow, Oregon Regional Primate Research Center, Beaverton, OR, and M. J. Stampfer, Harvard School of Public Health, Cambridge, MA.

mocysteinemia (Miller et al. 1992). In contrast, folate and vitamin B-12 deficiency may result in considerable hyperhomocysteinemia, which is rapidly normalized after replenishment with the deficient vitamin (Allen et al. 1990, Brattström et al. 1988a, Kang et al. 1987, Stabler et al. 1988). Even within their normal ranges,

Source: Ex. 1063 at 1276S; Paper 48 at 14.

State of the Art

Brattström

VITAMINS AS HOMOCYSTEINE-LOWERING AGENTS 1277S

Vitamins for lowering basal homocysteine concentration

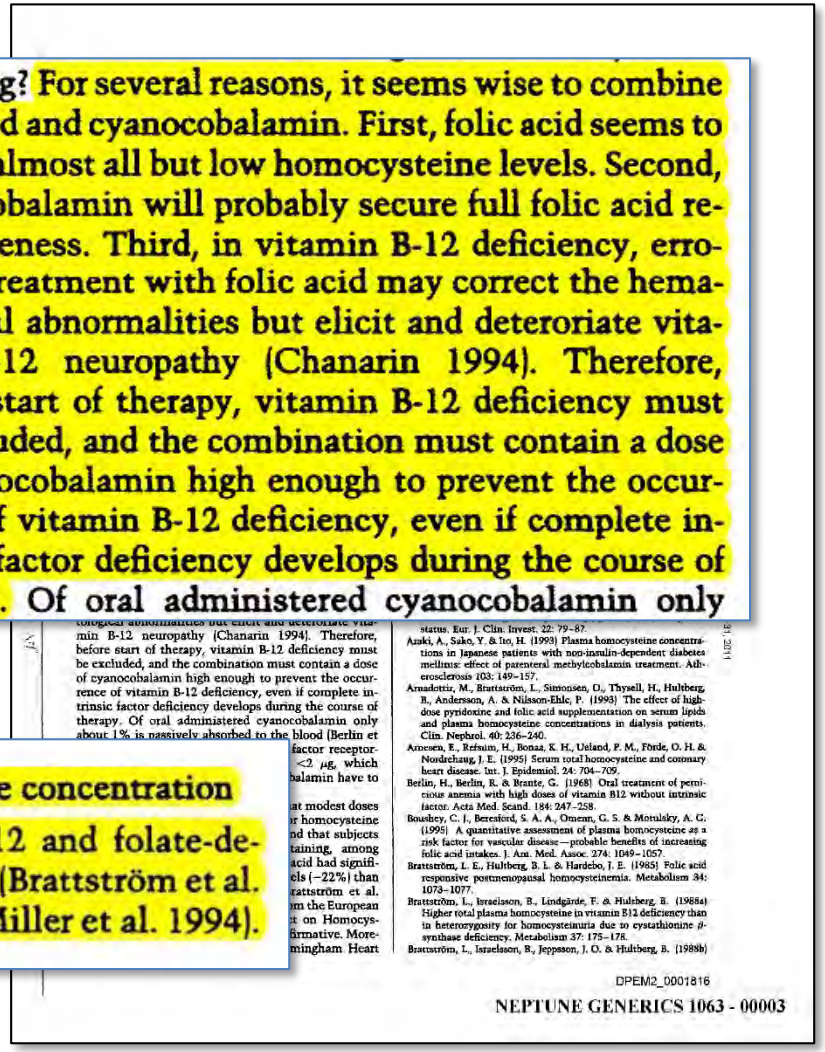
Renal insufficiency results both in moderate hyperhomocysteinemia and accelerated atherosclerosis (Wilcken et al. 1988). Several studies have consistently shown that oral treatment with folic acid (5–10 mg/day) reduces renal hyperhomocysteinemia by a mean of 30–60% (Arnadottier et al. 1993, Chauveau et al. 1994, Janssen et al. 1994, Wilcken et al. 1981, Wilcken et al. 1988). Oral pyridoxine has no homocysteine-lowering effect (Arnadottir et al. 1993, Wilcken et al. 1981).

In two studies, including a total of 28 nonvitamin-deficient healthy subjects with mostly normal plasma homocysteine concentrations, we tested the homocysteine-lowering effect of folic acid (5 mg/d for 2–4 wk) (Brattström et al. 1985, Brattström et al. 1988b). All but two with low homocysteine concentrations responded to folic acid, with reductions on average >30% and were most marked in those with high homocysteine concentrations. Oral treatment over 2 wk with pyridoxine (40 mg/d) or cyanocobalamin (1 mg/d) had no homocysteine-lowering effect (Brattström et al. 1988b). In another study, pyridoxine (120 mg/d for 6 wk) had no effect on plasma homocysteine concentration in 16 healthy subjects (Brattström and Hultberg unpublished). On the basis of these observations, we proposed that the homocysteine-lowering effect of folic acid in nonfolate-deficient subjects is that excess folic acid after conversion to methyltetrahydrofolate increases the rate by which homocysteine is remethylated to methionine. In contrast, excess vitamin B-12 and pyridoxine will not decrease plasma homocysteine unless deficiency is present because these vitamins serve as coenzymes and not as cosubstrates as does methyltetrahydrofolate (Brattström et al. 1988b).

Subsequently, we studied the effect of folic acid and pyridoxine in 20 moderately hyperhomocysteinemic patients with cardiovascular disease (Brattström et al. 1990). After pyridoxine (240 mg/d, for 2 wk) plasma homocysteine tended to increase, but after another 2 wk on pyridoxine with the addition of folic acid (10 mg/d) all patients showed reduced homocysteine concentrations, with 57% mean reduction. We also failed to show a homocysteine-lowering effect of high dose pyridoxine (300 mg/d for 12 wk) in 37 stroke patients (Lindgren, Brattström and Hultberg unpublished). In two recent studies of patients with vascular disease and hyperhomocysteinemia (Glueck et al. 1995, van den Berg et al. 1994) and in one study of normal normohomocysteinemic subjects (Haglund et al. 1993), the combination of pyridoxine (100–250 mg/d) and folic acid (5–10 mg/d) reduced plasma homocysteine by a mean of 51, 38, and 30%, respectively.

In groups of consecutive patients with acute myocardial infarction of whom most were normohomocysteinemic and all of whom had normal serum folate concentrations, we found that 2.5 and 10 mg of folic acid over 6 wk had similar homocysteine-lowering effect; in both groups plasma homocysteine was reduced by a mean of 27% (Landgren et al. 1995). Reductions were seen in all but two patients, both with low homocysteine values. With a few exceptions the response to folic acid was proportional to the pretreatment homocysteine levels. These exceptional patients were hyperhomocysteinemic and had low or low normal serum vitamin B-12 concentrations. In one with a subnormal vitamin B-12 concentration and a partial response to folic acid, oral treatment with cyanocobalamin (2 mg/d for 2 wk) normalized plasma homocysteine.

Hyperhomocysteinemia due to vitamin B-12 deficiency does not respond to folic acid therapy (Allen et al. 1990). It is likely, that even in subjects with low normal vitamin B-12 concentrations full response to folic acid cannot be achieved unless vitamin B-12 is given concomitantly (Landgren et al. 1995). This view is supported by recent studies by Ubbink et al. (1993a, 1993b, 1994). It was shown that men with moderate hyperhomocysteinemia (>16.3 μmol/l) in most cases had suboptimal plasma vitamin B-12 (<200 pmol/l) and folate (<5 nmol/l) concentrations (Ubbink et al. 1993a). Such men were in a 6-wk trial given either folic acid (0.65 mg/d), pyridoxine (10 mg/d), cyanocobalamin (0.4 mg/d) or the combination of these vitamins (Ubbink et al. 1994). Most but not all responded to folic acid, with the mean homocysteine concentration decreased from 28.8 to 16.8 μmol/l (−42%), a posttreatment value, however, still above normal. Pyridoxine had no homocysteine-lowering effect, whereas cyanocobalamin decreased plasma homocysteine by a mean of 15%. In contrast, all responded to the combination by a mean homocysteine reduction of 50% although homocysteine values were not normalized in all subjects during this short trial. Because the majority of these men probably had suboptimal vitamin B-12 status, homocysteine lowering could have been better if a higher cyanocobalmin dose had been used or if the treatment period had been extended for several weeks. There are recent results showing that high dose parenteral administration of cobalamin decreases plasma homocysteine in



lowering? For several reasons, it seems wise to combine folic acid and cyanocobalamin. First, folic acid seems to reduce almost all but low homocysteine levels. Second, cyanocobalamin will probably secure full folic acid responsiveness. Third, in vitamin B-12 deficiency, erroneous treatment with folic acid may correct the hematological abnormalities but elicit and deteroriate vitamin B-12 neuropathy (Chanarin 1994). Therefore, before start of therapy, vitamin B-12 deficiency must be excluded, and the combination must contain a dose of cyanocobalamin high enough to prevent the occurrence of vitamin B-12 deficiency, even if complete intrinsic factor deficiency develops during the course of therapy. Of oral administered cyanocobalamin only

an abnormally high basal homocysteine concentration mainly reflects impaired vitamin B-12 and folate-dependent homocysteine remethylation (Brattström et al. 1990, Christensen and Ueland 1993, Miller et al. 1994).

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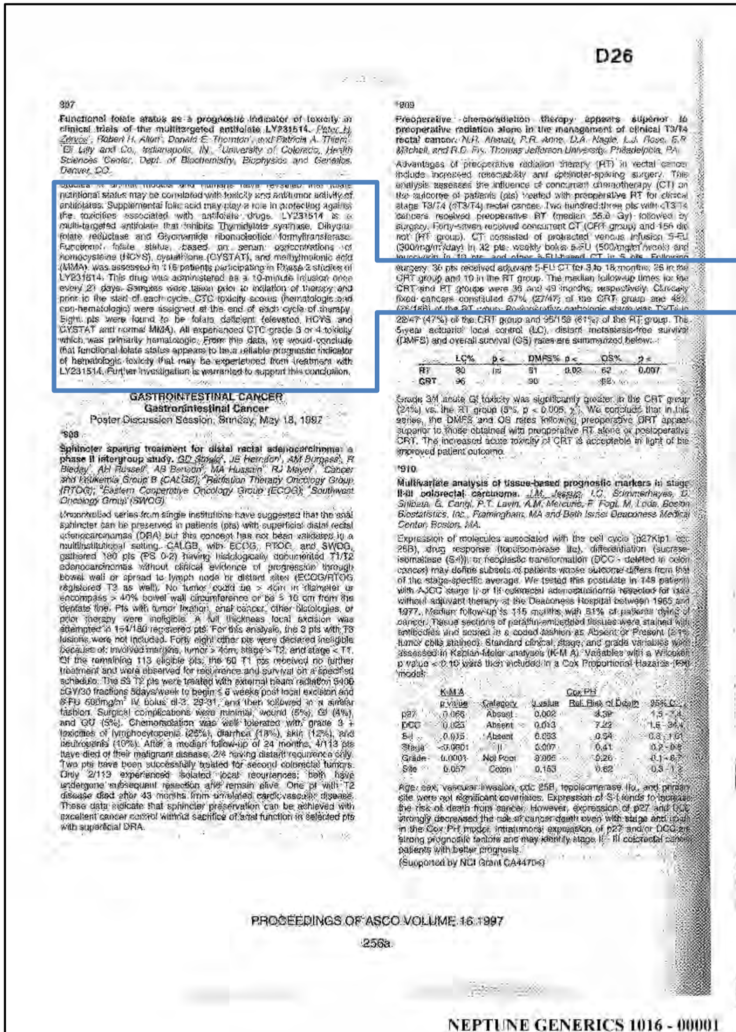
State of the Art

Zervos


D26

GASTROINTESTINAL CANCER

Gastrointestinal Cancer

Poster Discussion Session, Sunday, May 18, 1997

PROCEEDINGS OF ASCO VOLUME 16 1997

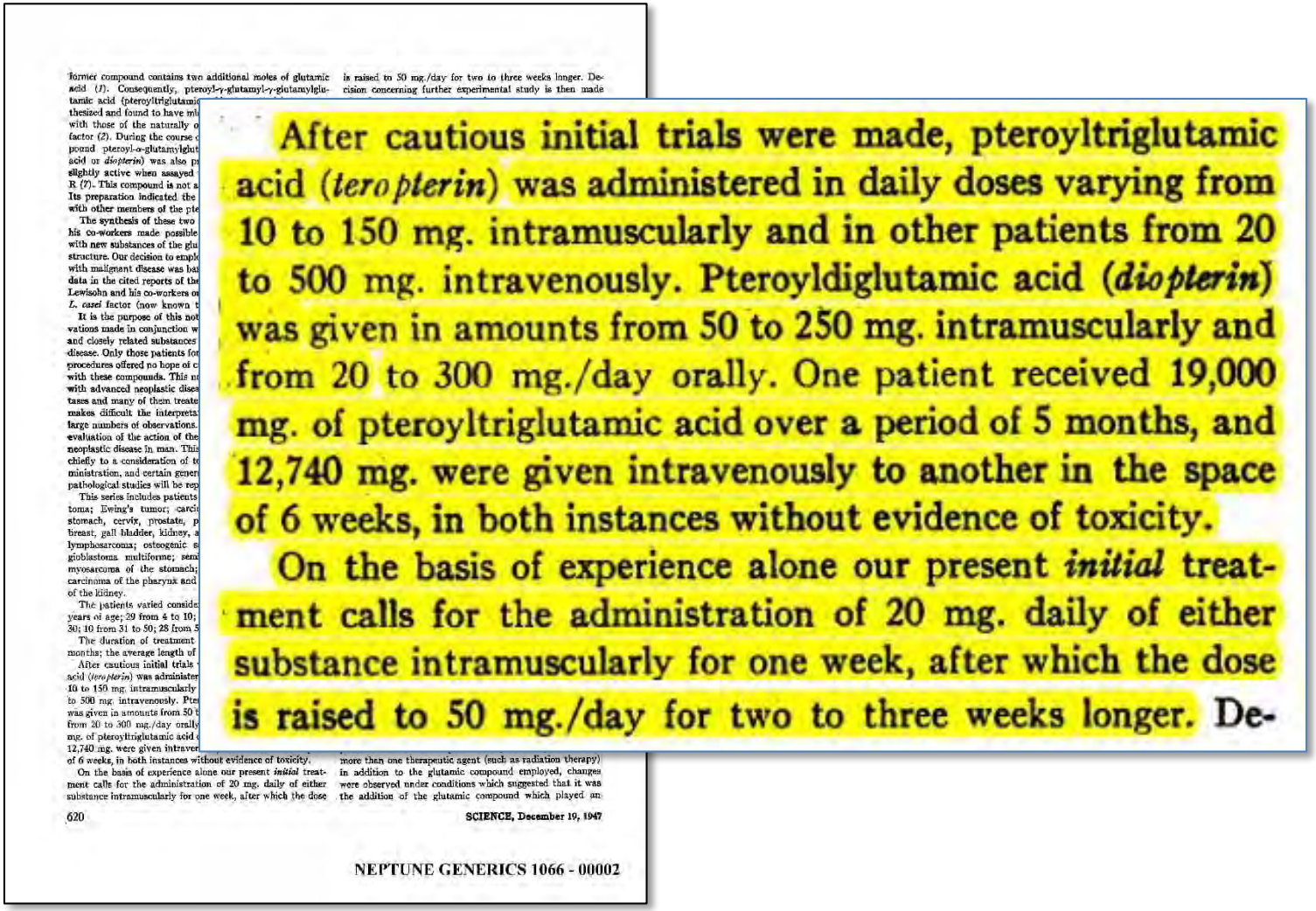
256a

NEPTUNE GENERICS 1016 - 00001

Studies in animal models and humans have revealed that folate nutritional status may be correlated with toxicity and antitumor activity of antifolates. Supplemental folic acid may play a role in protecting against the toxicities associated with antifolate drugs. LY231514 is a multi-targeted antifolate that inhibits Thymidylate synthase, Dihydrofolate reductase and Glycinamide ribonucleotide formyltransferase. Functional folate status, based on serum concentrations of homocysteine (HCYS), cystathione (CYSTAT), and methylmalonic acid (MMA), was assessed in 116 patients participating in Phase 2 studies of LY231514. This drug was administered as a 10-minute infusion once every 21 days. Samples were taken prior to initiation of therapy and prior to the start of each cycle. CTC toxicity scores (hematologic and non-hematologic) were assigned at the end of each cycle of therapy. Eight pts were found to be folate deficient (elevated HCYS and CYSTAT and normal MMA). All experienced CTC grade 3 or 4 toxicity which was primarily hematologic. From this data, we would conclude that functional folate status appears to be a reliable prognostic indicator of hematologic toxicity that may be experienced from treatment with LY231514. Further investigation is warranted to support this conclusion.

State of the Art

Farber 1947



After cautious initial trials were made, pteroyltriglutamic acid (*teropterin*) was administered in daily doses varying from 10 to 150 mg. intramuscularly and in other patients from 20 to 500 mg. intravenously. Pteroyldiglutamic acid (*dio pterin*) was given in amounts from 50 to 250 mg. intramuscularly and from 20 to 300 mg./day orally. One patient received 19,000 mg. of pteroyltriglutamic acid over a period of 5 months, and 12,740 mg. were given intravenously to another in the space of 6 weeks, in both instances without evidence of toxicity.

On the basis of experience alone our present *initial* treatment calls for the administration of 20 mg. daily of either substance intramuscularly for one week, after which the dose is raised to 50 mg./day for two to three weeks longer. De-

former compound contains two additional moles of glutamic acid (*1*). Consequently, pteroyl-γ-glutamyl-γ-glutamylglutamic acid (pteroyltriglutamic
thesized and found to have
with those of the naturally
factor (*2*). During the course
pound pteroyl-α-glutamylglut
acid or *dio pterin*) was also p
slightly active when assayed
R (*7*). This compound is not a
Its preparation indicated the
with other members of the pte
The synthesis of these two
his co-workers made possible
with new substances of the glu
structure. Our decision to empl
with malignant disease was ba
data in the cited reports of the
Lewisohn and his co-workers o
L. casei factor (now known
It is the purpose of this not
vations made in conjunction w
and closely related substances
disease. Only those patients for
procedures offered no hope of c
with these compounds. This n
with advanced neoplastic disea
tases and many of them treate
makes difficult the interpreta
large numbers of observations.
evaluation of the action of the
neoplastic disease in man. This
chiefly to a consideration of t
ministration, and certain gener
pathological studies will be rep
This series includes patients
toma; Ewing's tumor; carci
stomach, cervix, prostate, p
breast, gall bladder, kidney, a
lymphosarcoma; osteogenic s
glioblastoma multiforme; sem
myosarcoma of the stomach;
carcinoma of the pharynx and
of the kidney.
The patients varied consid
years of age; 29 from 4 to 10;
30; 10 from 31 to 50; 28 from 5
The duration of treatment
months; the average length of
After cautious initial trials
acid (*teropterin*) was administer
10 to 150 mg. intramuscularly
to 500 mg. intravenously. Pte
was given in amounts from 50 t
from 20 to 300 mg./day orally
mg. of pteroyltriglutamic acid
12,740 mg. were given intrave
of 6 weeks, in both instances without evidence of toxicity.
On the basis of experience alone our present *initial* treatment calls for the administration of 20 mg. daily of either substance intramuscularly for one week, after which the dose

more than one therapeutic agent (such as radiation therapy) in addition to the glutamic compound employed, changes were observed under conditions which suggested that it was the addition of the glutamic compound which played an

620

SCIENCE, December 19, 1947

NEPTUNE GENERICS 1066 - 00002

Source: Ex. 1066 at 620; Paper 48 at 17.

State of the Art

Farber 1948

The New England
Journal of Medicine

Volume 238 JUNE 3, 1948 Number 23

TEMPORARY REMISSIONS IN ACUTE LEUKEMIA IN CHILDREN PRODUCED BY FOLIC ACID ANTAGONIST, 4-AMINOPTEROYL-GLUTAMIC ACID (AMINOPTERIN)*

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ROBERT F. SYLVESTER, JR., M.D.,¶ AND JAMES A. WOLFF, M.D.‖
BOSTON

IT IS the purpose of this paper to record the results of clinical and hematologic studies on 5 children with acute leukemia treated by the intramuscular injection of a synthetic compound, 4-aminopteroylglutamic acid (aminopterin). This substance is an antagonist to folic acid regarding the growth of *Streptococcus faecalis R.*

The occurrence of what he interpreted as an "acceleration phenomenon" in the leukemic process as seen in the marrow and viscera of children with acute leukemia treated by the injection of folic acid conjugates[1] — pteroyltriglutamic acid (teropterin) and pteroyldiglutamic acid (diopterin) — and an experience gained from studies on folic acid deficiency suggested to Farber that folic acid antagonists might be of value in the treatment of patients with acute leukemia.[2] Post-mortem studies of leukemic infiltrates of the bone marrow and viscera in patients treated with folic acid conjugates were regarded by Farber as evidences of an acceleration of the leukemic processes to a degree not encountered in his experience with some 200 post-mortem examinations on children with acute leukemia not so treated. It appeared worth while, therefore, to ascertain if this acceleration phenomenon could be employed to advantage either by radiation or nitrogen mustard therapy after pretreatment with folic acid conjugates or by the administration of antagonists to folic acid.[2] A series of folic acid antagonists was made available by Dr. Y. Subbarow and his colleagues.[3–5]

The objective data sufficient to justify research in the direction of antagonists to folic acid in the treatment of leukemia were obtained from studies on a four-year-old girl with a rapidly progressing acute myelogenous leukemia.[2] Treatment from February 17 to March 24, 1947, with pteroyldiglutamic acid (diopterin), in a dosage of 100 to 300 mg. intramuscularly daily, had no effect upon the hematologic picture. The patient appeared to be moribund. A second bone-marrow biopsy on March 25 verified the diagnosis of myelogenous leukemia. Pteroylaspartic acid, the first antagonist to folic acid to be employed in our studies, was given intramuscularly from March 28 to April 4 in amounts of 40 mg. daily without altering the clinical course. Post-mortem examination on April 4 revealed a markedly hypoplastic bone marrow, with a few immature cells. A change of this magnitude in such a short time has not been encountered in the marrow of leukemic children in our experience.

This observation was followed by clinical, laboratory, and post-mortem studies** on a group of 14 children with acute leukemia treated with pteroylaspartic acid and on 7 treated with methylpteroic acid. The details of these observations will be reported separately.

Sufficient encouragement was obtained from these observations to justify further studies on the effect of more powerful antagonists to folic acid on the course of acute leukemia in children. Since November, 1947, when a sufficiently pure substance became available, to the time of this writing (April 15, 1948) we have made studies on 16 children with acute leukemia to whom the most powerful folic antagonist we have yet encountered, 4-aminopteroylglutamic acid (aminopterin††) was administered by intramuscular injection. Many of these children were moribund at the onset of therapy. Of 16 infants and children with acute leukemia treated with aminopterin 10 showed clinical, hematologic and pathological evidences of improvement of important

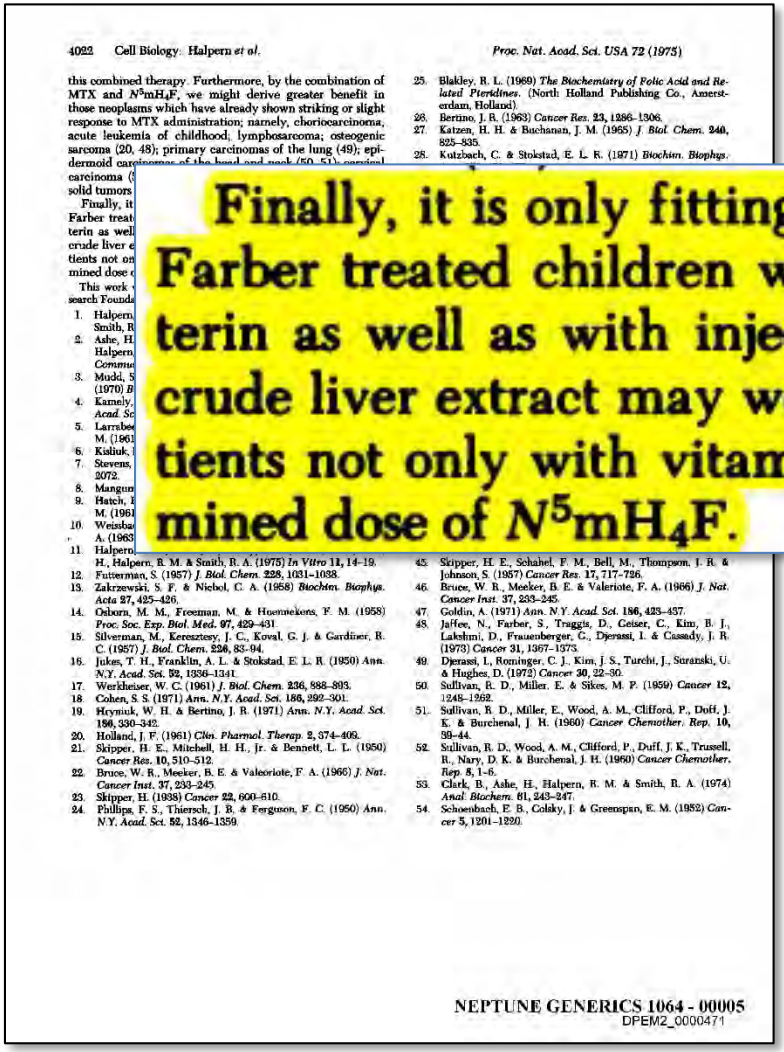
*Presented at a meeting of the Division of Laboratories and Research, The Children's Medical Center, Boston, April 8, 1948.
This study was supported in part under Grant No. 250 of the National Cancer Institute, United States Public Health Service, and in part under a grant from the Charles H. Hood Dairy Foundation.
†Assistant professor of pathology, Harvard Medical School; pathologist-in-chief and chairman, Division of Laboratories and Research, The Children's Medical Center, Boston.
‡Assistant professor of pediatrics, Harvard Medical School; hematologist and physician to The Children's Medical Center, Boston.
§Research fellow in pathology and tumor research, The Children's Medical Center, Boston.
¶Research fellow in pathology and tumor research, The Children's Medical Center, Boston.
‖Research fellow in pediatrics, The Children's Medical Center, Boston.
**These studies were carried out by a group consisting of Sidney Farber, Gilbert G. Lenz, James W. Hawkins, Ernst Eichwald, Robert D. Mercer and E. Cowperthwaite Peirce, II.
††This compound was first synthesized by the Calco Chemical Division of the American Cyanamid Company.

leukemia not so treated. It appeared worth while, therefore, to ascertain if this acceleration phenomenon could be employed to advantage either by radiation or nitrogen mustard therapy after pretreatment with folic acid conjugates or by the administration of antagonists to folic acid.[2] A series

Source: Ex. 2042 at 787; Paper 48 at 17.

State of the Art

Halpern



4022 Cell Biology: Halpern *et al.* *Proc. Nat. Acad. Sci. USA 72 (1975)*

this combined therapy. Furthermore, by the combination of MTX and N^5mH_4F, we might derive greater benefit in those neoplasms which have already shown striking or slight response to MTX administration; namely, choriocarcinoma, acute leukemia of childhood, lymphosarcoma; osteogenic sarcoma (20, 48); primary carcinomas of the lung (49); epidermoid carcinomas of the head and neck (50, 51); cervical carcinoma (
solid tumors
Finally, it
Farber treat
terin as well
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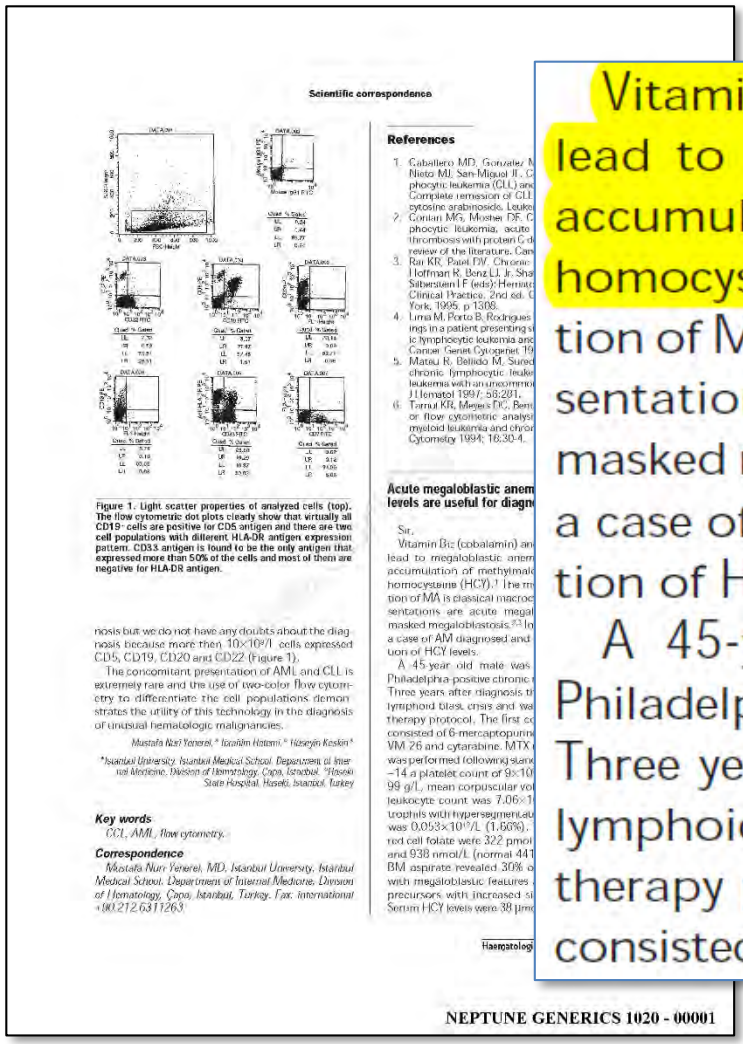
Finally, it is only fitting to recall that in 1948 Dr. Sidney Farber treated children with acute leukemia with aminopterin as well as with injections of crude liver extract. The crude liver extract may well have supplied these very ill patients not only with vitamin B_{12}, but also with an undetermined dose of N^5mH_4F.

State of the Art

Carrasco



Scientific correspondence

Figure 1. Light scatter properties of analyzed cells (top). The flow cytometric dot plots clearly show that virtually all CD19+ cells are positive for CD5 antigen and there are two cell populations with different HLA-DR antigen expression pattern. CD33 antigen is found to be the only antigen that expressed more than 50% of the cells and most of them are negative for HLA-DR antigen.

nosis but we do not have any doubts about the diag nosis because more then 10×10^9/l cells expressed CD5, CD19, CD20 and CD22 (Figure 1).

The concomitant presentation of AML and CLL is extremely rare and the use of two-color flow cytom etry to differentiate the cell populations demon strates the utility of this technology in the diagnosis of unusual hematologic malignancies.

Mustafa Nuri Yenerel,* Ibrahim Haberal,* Hüseyin Keskin*

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Key words
CCL, AML, flow cytometry.

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Acute megaloblastic anem levels are useful for diagn

Sir,

Vitamin B_{12} (cobalamin) an lead to megaloblastic anem accumulation of methylmalo homocysteine (HCY).[1] The m tion of MA is classical macroc sentations are acute megal masked megaloblastosis.[2,3] In a case of AM diagnosed and tion of HCY levels.

A 45-year old male was diagnosed as having Philadelphia-positive chronic myelogenous leukemia. Three years after diagnosis the patient developed a lymphoid blast crisis and was started on a chemo therapy protocol. The first consolidation treatment consisted of 6-mercaptopurine, methotrexate (MTX), VM 26 and cytarabine. MTX was performed following stand −14 a platelet count of $9\times10^$ 99 g/l, mean corpuscular vol leukocyte count was 7.06×1 trophils with hypersegmentat was 0.053×10^9/L (1.60%). red cell folate were 322 pmol and 938 nmol/L (normal 447 BM aspirate revealed 30% o with megaloblastic features precursers with increased si Serum HCY levels were 38 µm

Haematologi

NEPTUNE GENERICS 1020 - 00001

Vitamin B_{12} (cobalamin) and folic acid deficiencies lead to megaloblastic anemia (MA), and induce accumulation of methylmalonic acid (MMA) and homocysteine (HCY).[1] The most common presentation of MA is classical macrocytic anemia. Other presentations are acute megaloblastosis (AM) and masked megaloblastosis.[2,3] In this report, we present a case of AM diagnosed and followed up by evaluation of HCY levels.

A 45-year old male was diagnosed as having Philadelphia-positive chronic myelogenous leukemia. Three years after diagnosis the patient developed a lymphoid blast crisis and was started on a chemotherapy protocol. The first consolidation treatment consisted of 6-mercaptopurine, methotrexate (MTX),

State of the Art

Carrasco

Table 1. Evolution of analytical paramet
acid and vitamin B_{12} treatment.

	Pre-treatment Day –8	On Day
Platelets (x10⁹/L)	134	9
Leukocytes (x10⁹/L)	6.76	7.0
Hemoglobin (g/L)	91	9
MCV (fL)	93	9
Reticulocytes (x10¹²/L)	0.037	0.0
Homocysteine (µmol/L)	-	3

AM, acute megaloblastosis; MCV, mean corpuscular

increased.[9,10] HCY levels are also useful for AM follow-up of AM; levels return to normal after starting treatment with vitamin B_{12} or folic acid. The evaluation of

patient was diagnosed as having AM and began treatment with folinic acid 12 mg iv in one single dose and folic acid 5 mg/day po for 14 days and parenteral vitamin B_{12} 2 mg/day for 4 consecutive days. After 10 days of treatment the platelet count increased to 112×10^9/L and reticulocyte count to 0.163×10^{12}/L (5.41%). Vitamin B_{12} level was 716 pmol/L, red cell folate level 1,506 nmol/L and serum HCY level decreased to normal value (9 µmol/L) (Table 1).

Four different clinical forms of megaloblastosis have been described.[3,4] The classical form has an insidious onset with frequent neurologic symptoms and macrocytic anemia. Vitamin B_{12} and/or red cell folate levels are decreased. The second form is the subtle MA anemia with ill-defined clinical symptoms and decreased or borderline vitamin B_{12} and folic acid levels with other abnormalities (dUST, HCY, MMA).[2] Masked megaloblastosis coexists with other deficiencies; MCV is normal or decreased.[5,6] MA of acute onset is the rarest form.[3] There are two clinical presentations; the masked undiagnosed classical MA with cytopenias of abrupt onset and the so called AM.[3,7] In AM severe thrombocytopenia develops in 1 to 3 weeks, MCV is normal or only moderately increased. This presentation is more frequent in patients with risk factors: parenteral nutrition, infection, dialysis or treatment with some antifolate drugs. Mortality is high.[3] The reticulocyte count is low. Vitamin B_{12} and red cell folate levels are normal. BM aspirate shows megaloblastic changes. Classically, dUST is used as a diagnostic test. Nevertheless, HCY serum assays provide a sensitive test for the diagnosis of AM, especially in its early stages.[3] In vitamin B_{12} deficiences both HCY and MMA levels are high. In

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State of the Art

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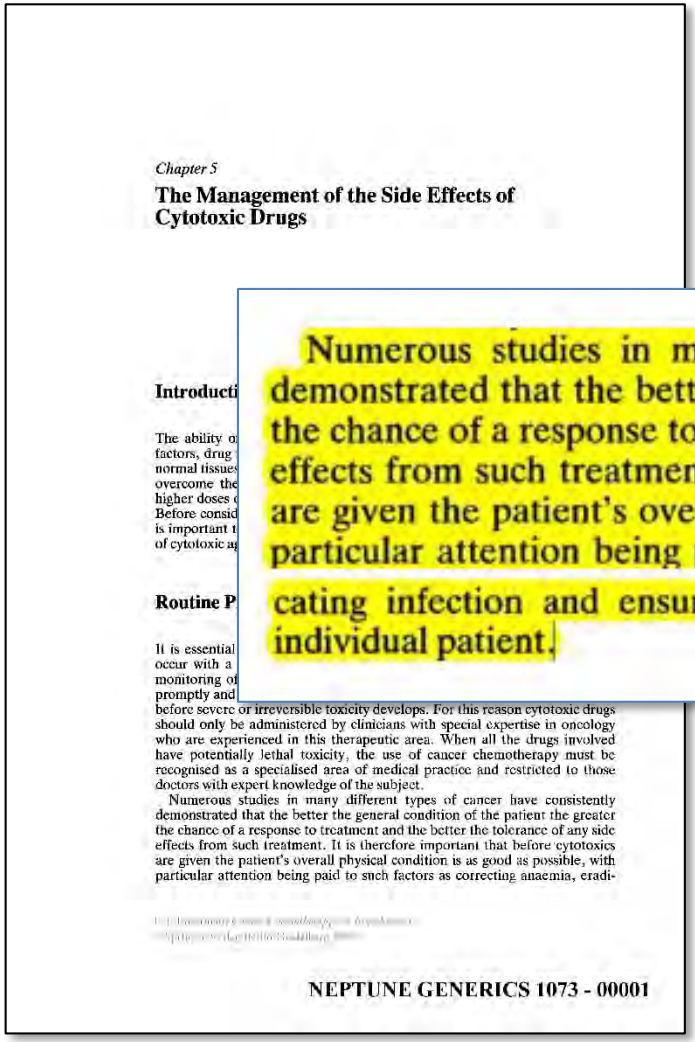

Chapter 5

The Management of the Side Effects of Cytotoxic Drugs

Introducti

The ability o
factors, drug
normal tissues
overcome the
higher doses
Before consid
is important t
of cytotoxic a

Routine P

It is essential
occur with a
monitoring of
promptly and
before severe or irreversible toxicity develops. For this reason cytotoxic drugs should only be administered by clinicians with special expertise in oncology who are experienced in this therapeutic area. When all the drugs involved have potentially lethal toxicity, the use of cancer chemotherapy must be recognised as a specialised area of medical practice and restricted to those doctors with expert knowledge of the subject.

Numerous studies in many different types of cancer have consistently demonstrated that the better the general condition of the patient the greater the chance of a response to treatment and the better the tolerance of any side effects from such treatment. It is therefore important that before cytotoxics are given the patient's overall physical condition is as good as possible, with particular attention being paid to such factors as correcting anaemia, eradi-

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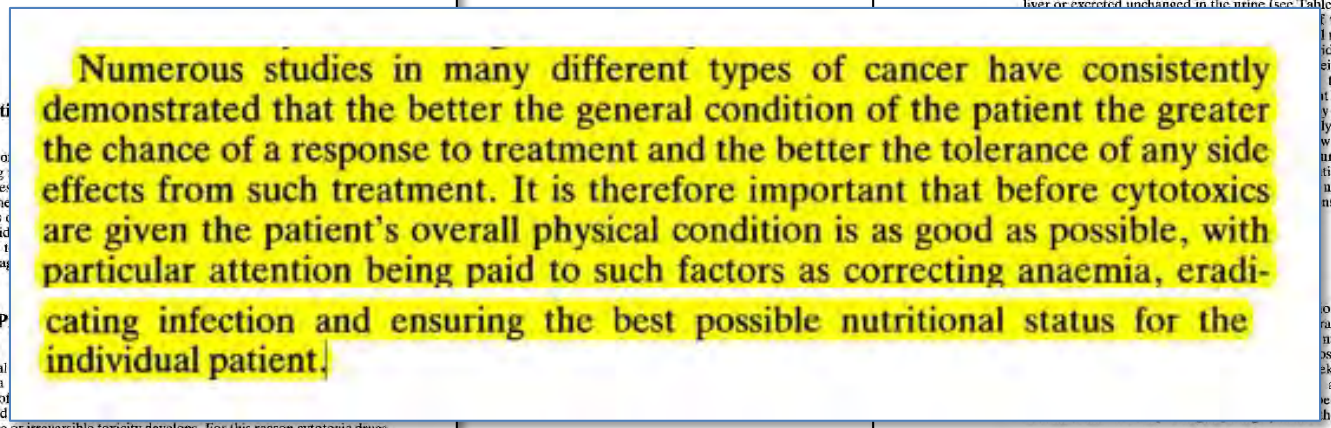
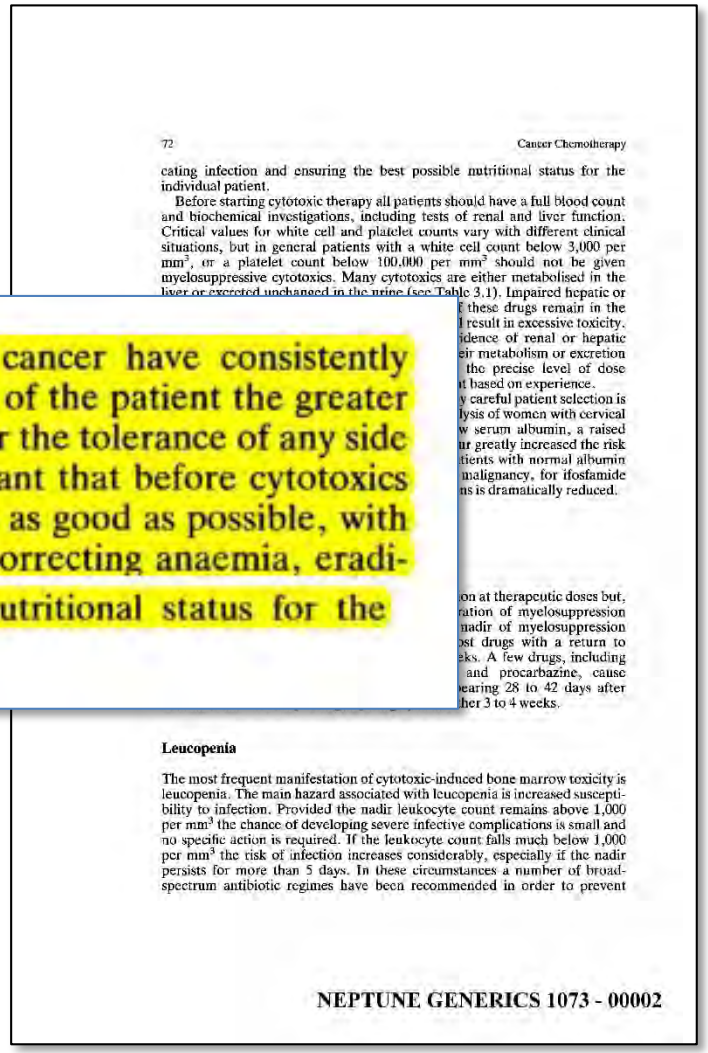


Numerous studies in many different types of cancer have consistently demonstrated that the better the general condition of the patient the greater the chance of a response to treatment and the better the tolerance of any side effects from such treatment. It is therefore important that before cytotoxics are given the patient's overall physical condition is as good as possible, with particular attention being paid to such factors as correcting anaemia, eradicating infection and ensuring the best possible nutritional status for the individual patient.



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cating infection and ensuring the best possible nutritional status for the individual patient.

Before starting cytotoxic therapy all patients should have a full blood count and biochemical investigations, including tests of renal and liver function. Critical values for white cell and platelet counts vary with different clinical situations, but in general patients with a white cell count below 3,000 per mm^3, or a platelet count below 100,000 per mm^3 should not be given myelosuppressive cytotoxics. Many cytotoxics are either metabolised in the liver or excreted unchanged in the urine (see Table 3.1). Impaired hepatic or

these drugs remain in the
result in excessive toxicity.
idence of renal or hepatic
eir metabolism or excretion
the precise level of dose
t based on experience.
careful patient selection is
lysis of women with cervical
w serum albumin, a raised
ur greatly increased the risk
tients with normal albumin
malignancy, for ifosfamide
ns is dramatically reduced.

on at therapeutic doses but,
ration of myelosuppression
nadir of myelosuppression
ost drugs with a return to
eks. A few drugs, including
and procarbazine, cause
earing 28 to 42 days after
her 3 to 4 weeks.

Leucopenia

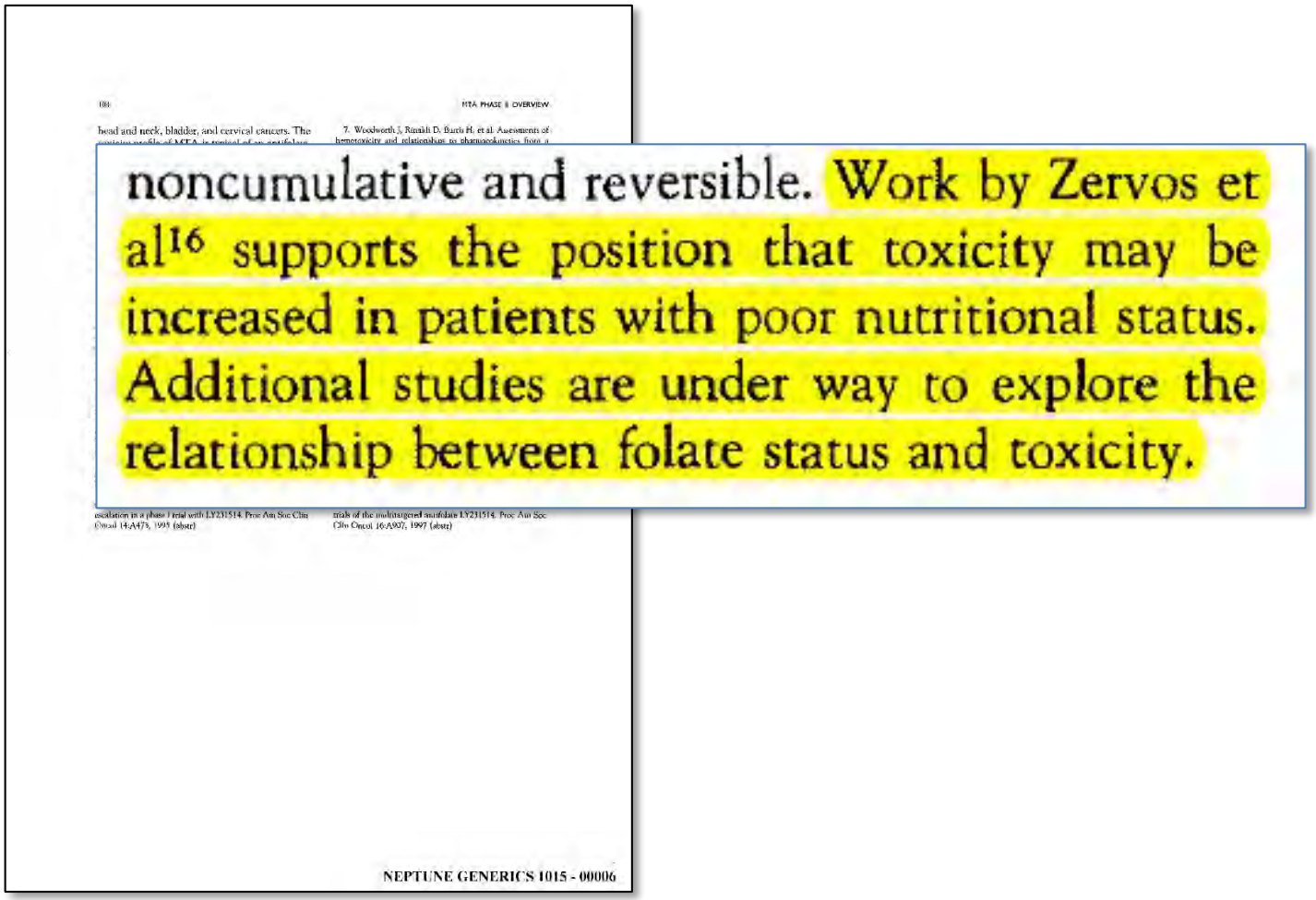
The most frequent manifestation of cytotoxic-induced bone marrow toxicity is leucopenia. The main hazard associated with leucopenia is increased susceptibility to infection. Provided the nadir leukocyte count remains above 1,000 per mm^3 the chance of developing severe infective complications is small and no specific action is required. If the leukocyte count falls much below 1,000 per mm^3 the risk of infection increases considerably, especially if the nadir persists for more than 5 days. In these circumstances a number of broad-spectrum antibiotic regimes have been recommended in order to prevent

NEPTUNE GENERICS 1073 - 00002

Source: Ex. 1073 at 71-72; Paper 48 at 18-19.

State of the Art

O' Dwyer



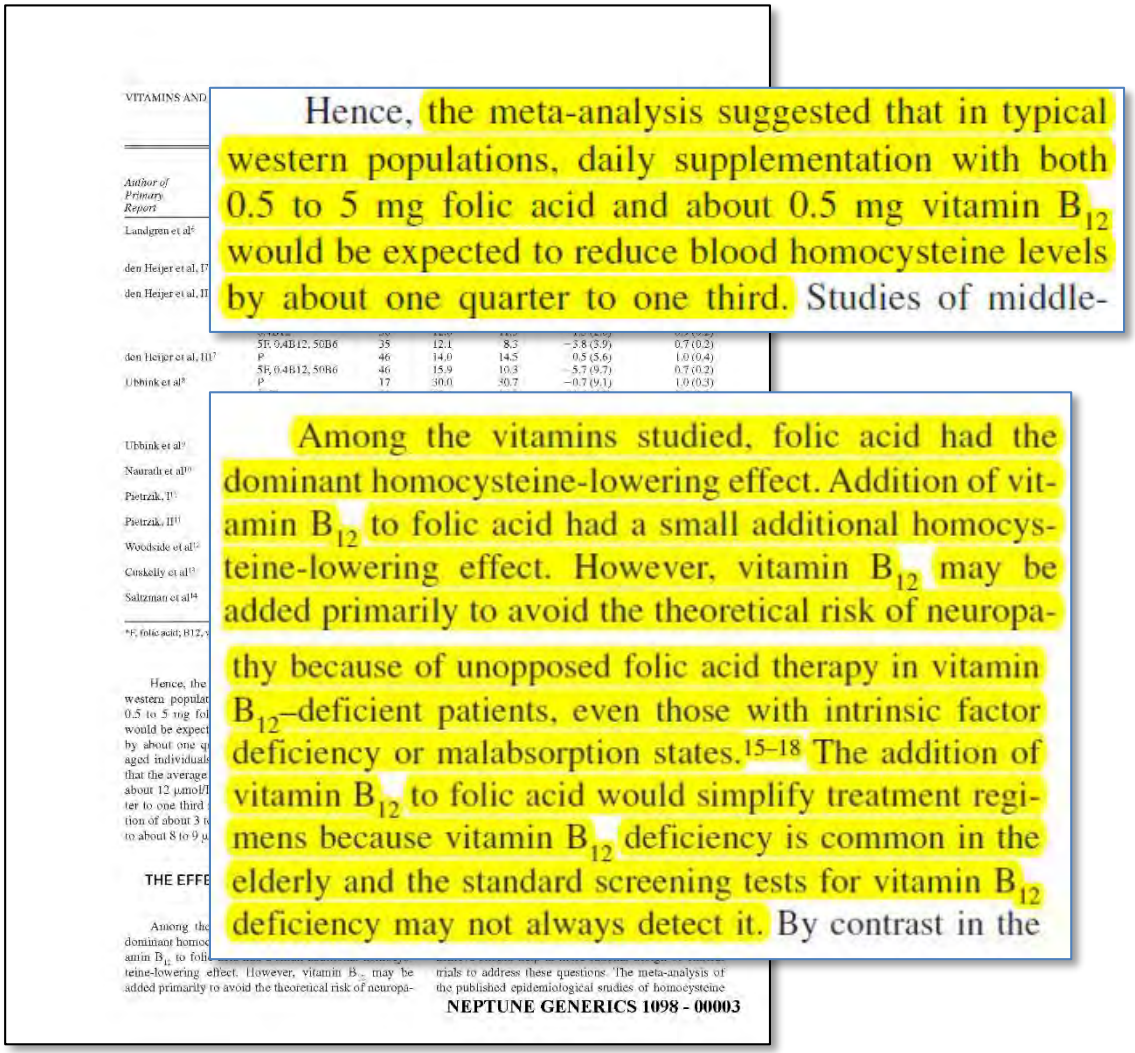
noncumulative and reversible. Work by Zervos et al[16] supports the position that toxicity may be increased in patients with poor nutritional status. Additional studies are under way to explore the relationship between folate status and toxicity.

NEPTUNE GENERICS 1015 - 00006

Source: Ex. 1015 at 104; Paper 48 at 20.

State of the Art

Clarke



Hence, the meta-analysis suggested that in typical western populations, daily supplementation with both 0.5 to 5 mg folic acid and about 0.5 mg vitamin B_{12} would be expected to reduce blood homocysteine levels by about one quarter to one third. Studies of middle-

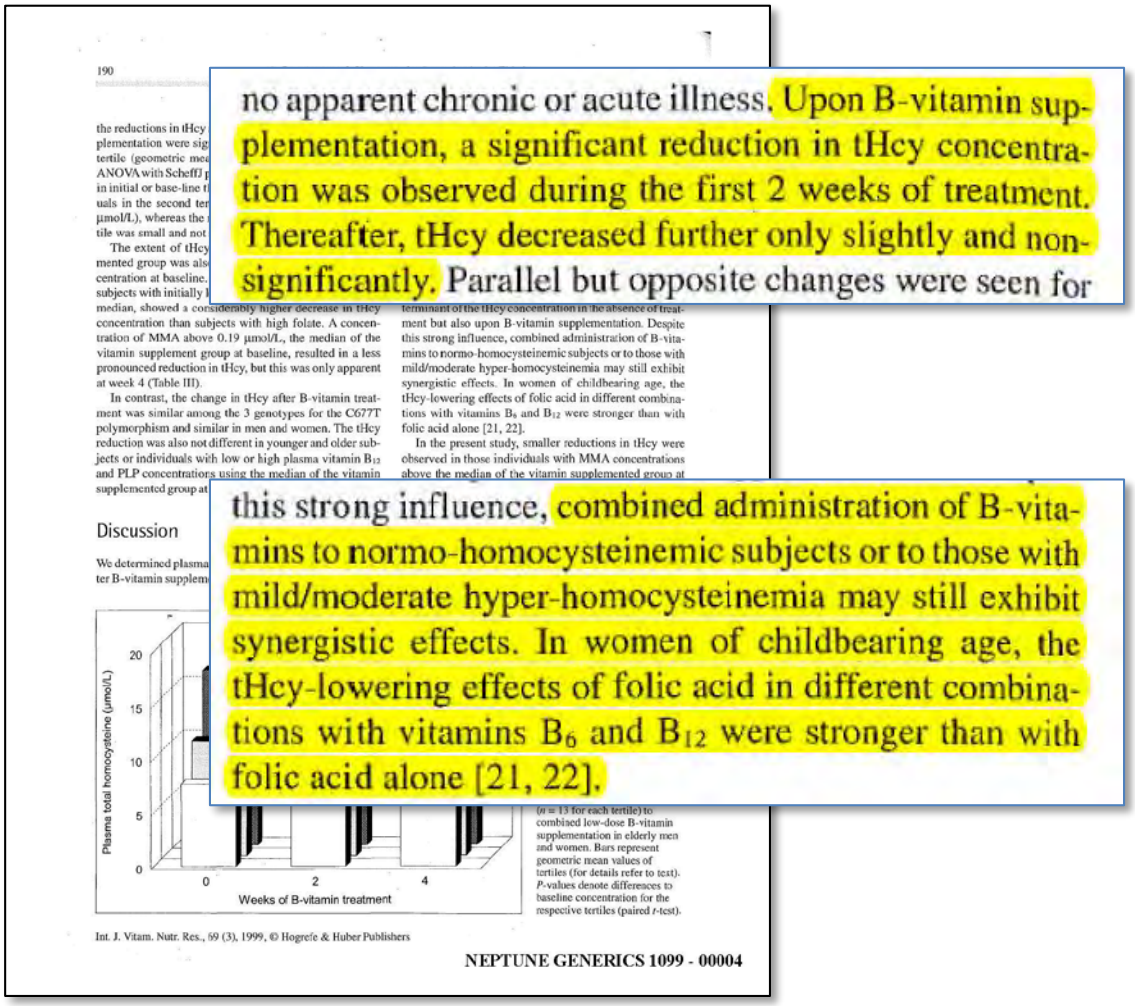
Among the vitamins studied, folic acid had the dominant homocysteine-lowering effect. Addition of vitamin B_{12} to folic acid had a small additional homocysteine-lowering effect. However, vitamin B_{12} may be added primarily to avoid the theoretical risk of neuropathy because of unopposed folic acid therapy in vitamin B_{12}–deficient patients, even those with intrinsic factor deficiency or malabsorption states.[15–18] The addition of vitamin B_{12} to folic acid would simplify treatment regimens because vitamin B_{12} deficiency is common in the elderly and the standard screening tests for vitamin B_{12} deficiency may not always detect it. By contrast in the

NEPTUNE GENERICS 1098 - 00003

Source: Ex. 1098 at 343; Paper 48 at 20.

State of the Art

Bronstrup II



190

the reductions in tHcy
plementation were sig
tertile (geometric mea
ANOVA with Scheffé p
in initial or base-line t
uals in the second ter
µmol/L), whereas the
tile was small and not

The extent of tHcy
mented group was als
centration at baseline.
subjects with initially
median, showed a considerably higher decrease in tHcy concentration than subjects with high folate. A concentration of MMA above 0.19 µmol/L, the median of the vitamin supplement group at baseline, resulted in a less pronounced reduction in tHcy, but this was only apparent at week 4 (Table III).

In contrast, the change in tHcy after B-vitamin treatment was similar among the 3 genotypes for the C677T polymorphism and similar in men and women. The tHcy reduction was also not different in younger and older subjects or individuals with low or high plasma vitamin B_{12} and PLP concentrations using the median of the vitamin supplemented group at

terminant of the tHcy concentration in the absence of treatment but also upon B-vitamin supplementation. Despite this strong influence, combined administration of B-vitamins to normo-homocysteinemic subjects or to those with mild/moderate hyper-homocysteinemia may still exhibit synergistic effects. In women of childbearing age, the tHcy-lowering effects of folic acid in different combinations with vitamins B_6 and B_{12} were stronger than with folic acid alone [21, 22].

In the present study, smaller reductions in tHcy were observed in those individuals with MMA concentrations above the median of the vitamin supplemented group at

Discussion

We determined plasma
ter B-vitamin supplem

(n = 13 for each tertile) to combined low-dose B-vitamin supplementation in elderly men and women. Bars represent geometric mean values of tertiles (for details refer to text). *P*-values denote differences to baseline concentration for the respective tertiles (paired *t*-test).

Int. J. Vitam. Nutr. Res., 69 (3), 1999, © Hogrefe & Huber Publishers

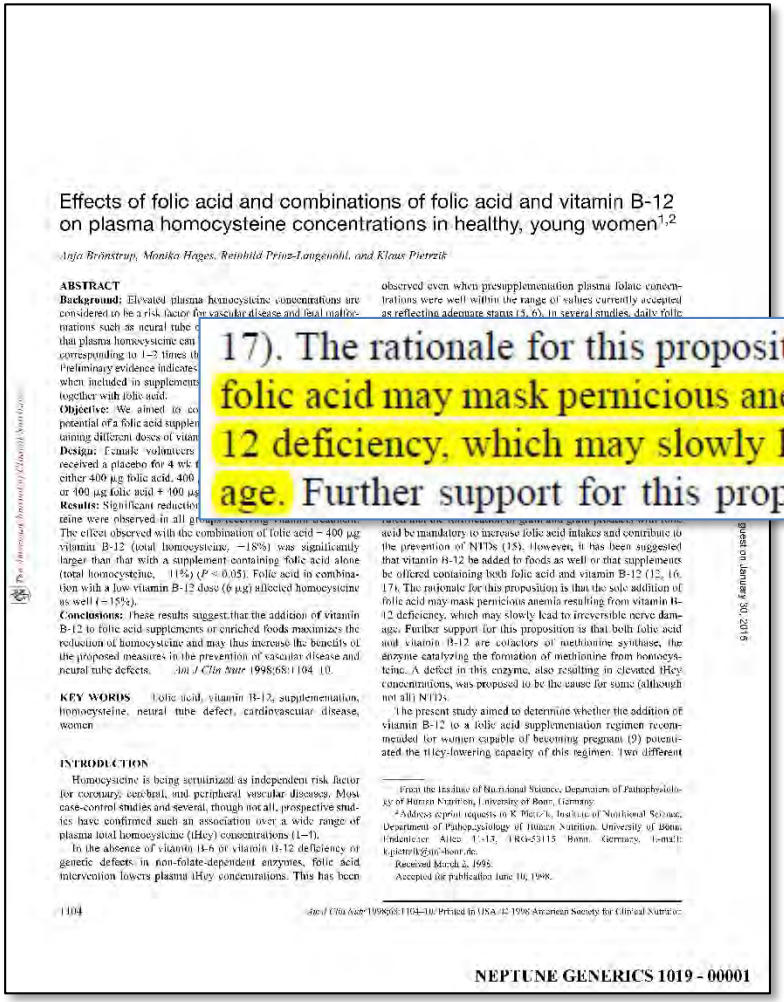
no apparent chronic or acute illness. Upon B-vitamin supplementation, a significant reduction in tHcy concentration was observed during the first 2 weeks of treatment. Thereafter, tHcy decreased further only slightly and non-significantly. Parallel but opposite changes were seen for

this strong influence, combined administration of B-vitamins to normo-homocysteinemic subjects or to those with mild/moderate hyper-homocysteinemia may still exhibit synergistic effects. In women of childbearing age, the tHcy-lowering effects of folic acid in different combinations with vitamins B_6 and B_{12} were stronger than with folic acid alone [21, 22].

Source: Ex. 1099 at 190; Paper 48 at 21.

State of the Art

Bronstrup I



Effects of folic acid and combinations of folic acid and vitamin B-12 on plasma homocysteine concentrations in healthy, young women[1,2]

Anja Bronstrup, Monika Hages, Reinhild Prinz-Langenohl, and Klaus Pietrzik

ABSTRACT

Background: Elevated plasma homocysteine concentrations are considered to be a risk factor for vascular disease and fetal malformations such as neural tube defects. Preliminary evidence indicates that plasma homocysteine can be lowered when included in supplements together with folic acid.

Objective: We aimed to compare the potential of a folic acid supplement containing different doses of vitamin B-12.

Design: Female volunteers received a placebo for 4 wk, then either 400 μg folic acid, 400 μg folic acid + 6 μg vitamin B-12, or 400 μg folic acid + 400 μg vitamin B-12 for 4 wk.

Results: Significant reductions in plasma homocysteine were observed in all groups receiving vitamin B-12. The effect observed with the combination of folic acid + 400 μg vitamin B-12 (total homocysteine, −18%) was significantly larger than that with a supplement containing folic acid alone (total homocysteine, −11%) ($P < 0.05$). Folic acid in combination with a low vitamin B-12 dose (6 μg) affected homocysteine as well (−15%).

Conclusions: These results suggest that the addition of vitamin B-12 to folic acid–supplements or enriched foods maximizes the reduction of homocysteine and may thus increase the benefits of the proposed measures in the prevention of vascular disease and neural tube defects. *Am J Clin Nutr* 1998;68:1104–10.

KEY WORDS Folic acid, vitamin B-12, supplementation, homocysteine, neural tube defect, cardiovascular disease, women

INTRODUCTION

Homocysteine is being scrutinized as independent risk factor for coronary, cerebral, and peripheral vascular diseases. Most case-control studies and several, though not all, prospective studies have confirmed such an association over a wide range of plasma total homocysteine (tHcy) concentrations (1–7).

In the absence of vitamin B-6 or vitamin B-12 deficiency or genetic defects in non-folate-dependent enzymes, folic acid intervention lowers plasma tHcy concentrations. This has been observed even when presupplementation plasma folate concentrations were well within the range of values currently accepted as reflecting adequate status (5, 6). In several studies, daily folic acid be mandatory to increase folic acid intakes and contribute to the prevention of NTDs (18). However, it has been suggested that vitamin B-12 be added to foods as well or that supplements be offered containing both folic acid and vitamin B-12 (12, 16, 17). The rationale for this proposition is that the sole addition of folic acid may mask pernicious anemia resulting from vitamin B-12 deficiency, which may slowly lead to irreversible nerve damage. Further support for this proposition is that both folic acid and vitamin B-12 are cofactors of methionine synthase, the enzyme catalyzing the formation of methionine from homocysteine. A defect in this enzyme, also resulting in elevated tHcy concentrations, was proposed to be the cause for some (although not all) NTDs.

The present study aimed to determine whether the addition of vitamin B-12 to a folic acid supplementation regimen recommended for women capable of becoming pregnant (9) potentiated the tHcy-lowering capacity of this regimen. Two different

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17). The rationale for this proposition is that the sole addition of folic acid may mask pernicious anemia resulting from vitamin B-12 deficiency, which may slowly lead to irreversible nerve damage. Further support for this proposition is that both folic acid

State of the Art

Bronstrup I

HOMOCYSTEINE LOWERING BY FOLIC ACID AND VITAMIN B-12 1109

In this study, vitamin B-12 supplementation increased the tHcy-lowering potential of folic acid; this was especially obvious

nmol/L. Because folate and vitamin B-12 have a synergistic function as cofactors of methionine synthase, sufficiency of both seems to be important to increase enzyme activity, whereas a higher availability of only one cofactor, especially in subjects with an already good supply of this cofactor, might lead to only a limited increase in enzyme activity.

could counter the higher prevalence of vitamin B-12 deficiency in the elderly and the possibility of masked pernicious anemia with supplementation of folic acid alone (16, 28, 30). Doses of

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Rinaldi II

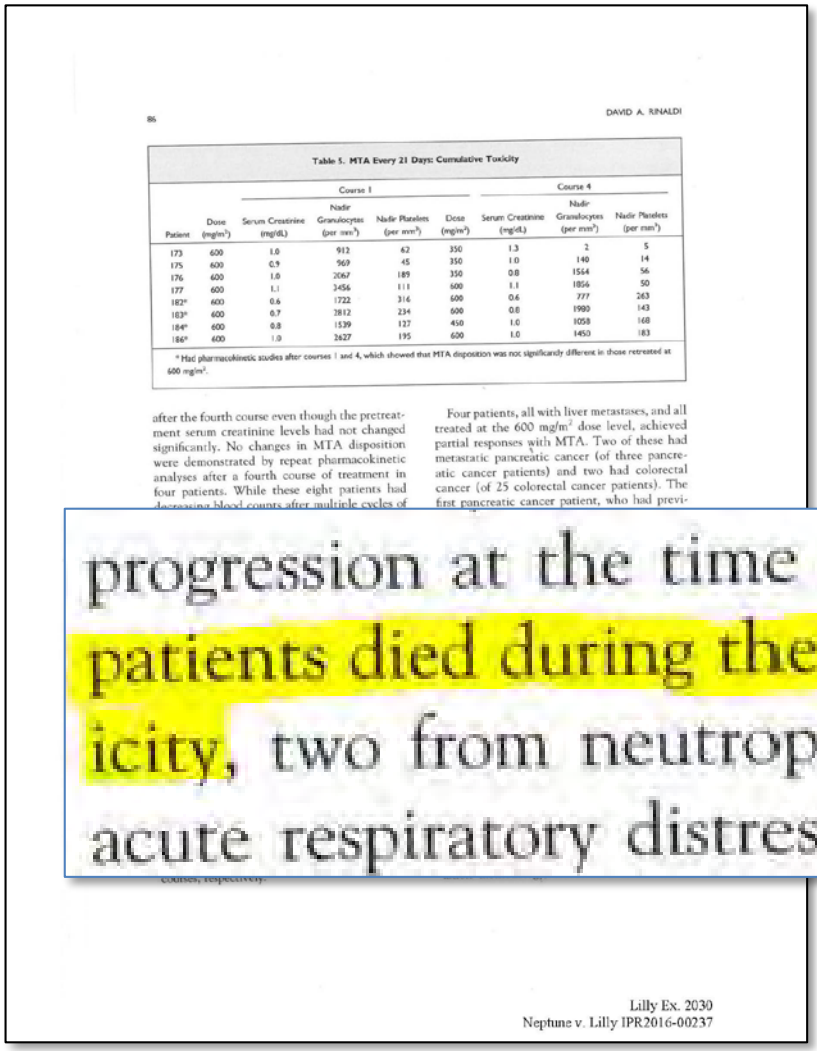

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Table 5. MTA Every 21 Days: Cumulative Toxicity

Patient	Course 1 Dose (mg/m²)	Course 1 Serum Creatinine (mg/dL)	Course 1 Nadir Granulocytes (per mm³)	Course 1 Nadir Platelets (per mm³)	Course 4 Dose (mg/m²)	Course 4 Serum Creatinine (mg/dL)	Course 4 Nadir Granulocytes (per mm³)	Course 4 Nadir Platelets (per mm³)
173	600	1.0	912	62	350	1.3	2	5
175	600	0.9	969	45	350	1.0	140	14
176	600	1.0	2067	189	350	0.8	1564	56
177	600	1.1	3456	111	600	1.1	1856	50
182*	600	0.6	1722	316	600	0.6	777	263
183*	600	0.7	2812	234	600	0.8	1980	143
184*	600	0.8	1539	127	450	1.0	1058	168
186*	600	1.0	2627	195	600	1.0	1450	183

* Had pharmacokinetic studies after courses 1 and 4, which showed that MTA disposition was not significantly different in those retreated at 600 mg/m².

after the fourth course even though the pretreatment serum creatinine levels had not changed significantly. No changes in MTA disposition were demonstrated by repeat pharmacokinetic analyses after a fourth course of treatment in four patients. While these eight patients had decreasing blood counts after multiple cycles of

Four patients, all with liver metastases, and all treated at the 600 mg/m² dose level, achieved partial responses with MTA. Two of these had metastatic pancreatic cancer (of three pancreatic cancer patients) and two had colorectal cancer (of 25 colorectal cancer patients). The first pancreatic cancer patient, who had previ-

courses, respectively.

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Neptune v. Lilly IPR2016-00237

progression at the time of discontinuation. Three patients died during the study related to drug toxicity, two from neutropenic sepsis, and one from acute respiratory distress syndrome. These deaths

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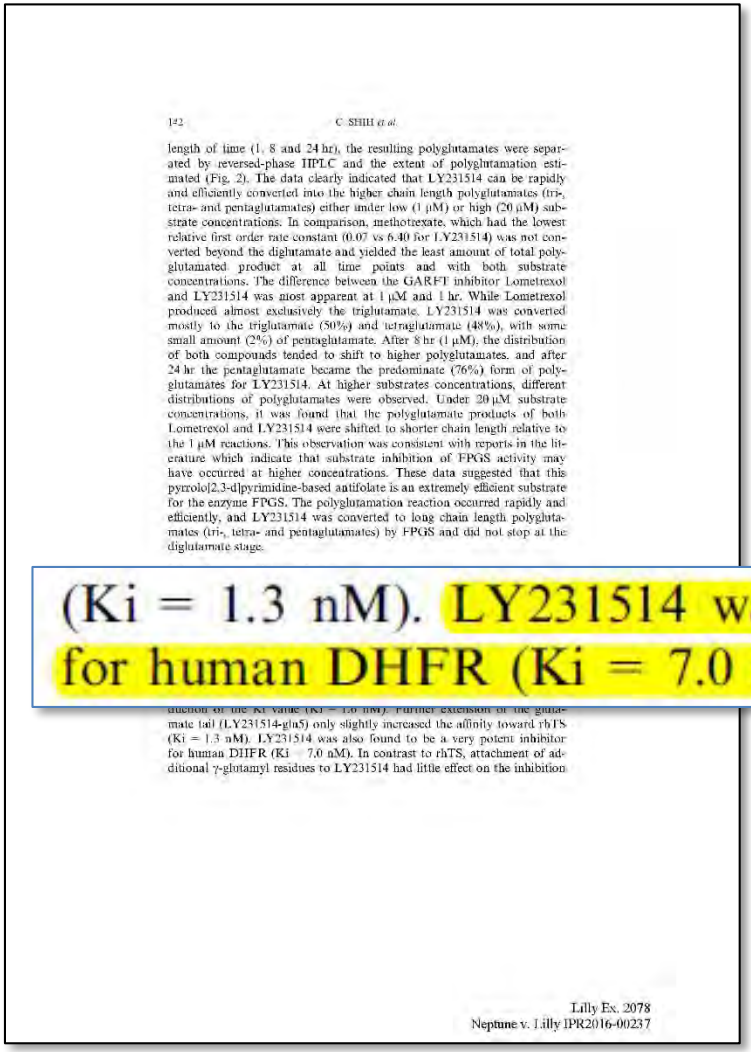

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length of time (1, 8 and 24 hr), the resulting polyglutamates were separated by reversed-phase HPLC and the extent of polyglutamation estimated (Fig. 2). The data clearly indicated that LY231514 can be rapidly and efficiently converted into the higher chain length polyglutamates (tri-, tetra- and pentaglutamates) either under low (1 μM) or high (20 μM) substrate concentrations. In comparison, methotrexate, which had the lowest relative first order rate constant (0.07 vs 6.40 for LY231514) was not converted beyond the diglutamate and yielded the least amount of total polyglutamated product at all time points and with both substrate concentrations. The difference between the GARFT inhibitor Lometrexol and LY231514 was most apparent at 1 μM and 1 hr. While Lometrexol produced almost exclusively the triglutamate, LY231514 was converted mostly to the triglutamate (50%) and tetraglutamate (48%), with some small amount (2%) of pentaglutamate. After 8 hr (1 μM), the distribution of both compounds tended to shift to higher polyglutamates, and after 24 hr the pentaglutamate became the predominate (76%) form of polyglutamates for LY231514. At higher substrates concentrations, different distributions of polyglutamates were observed. Under 20 μM substrate concentrations, it was found that the polyglutamate products of both Lometrexol and LY231514 were shifted to shorter chain length relative to the 1 μM reactions. This observation was consistent with reports in the literature which indicate that substrate inhibition of FPGS activity may have occurred at higher concentrations. These data suggested that this pyrrolo[2,3-d]pyrimidine-based antifolate is an extremely efficient substrate for the enzyme FPGS. The polyglutamation reaction occurred rapidly and efficiently, and LY231514 was converted to long chain length polyglutamates (tri-, tetra- and pentaglutamates) by FPGS and did not stop at the diglutamate stage.

duction of the Ki value (Ki = 1.6 nM). Further extension of the glutamate tail (LY231514-glu5) only slightly increased the affinity toward rhTS (Ki = 1.3 nM). LY231514 was also found to be a very potent inhibitor for human DHFR (Ki = 7.0 nM). In contrast to rhTS, attachment of additional γ-glutamyl residues to LY231514 had little effect on the inhibition

Lilly Ex. 2078
Neptune v. Lilly IPR2016-00237

(Ki = 1.3 nM). LY231514 was also found to be a very potent inhibitor for human DHFR (Ki = 7.0 nM). In contrast to rhTS, attachment of ad-

Source: Ex. 2078 at 142; Paper 48 at 13.

PDR 1999

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PHYSICIANS' DESK REFERENCE®

Methotrexate Sodium—Cont.

Folate deficiency states may increase methotrexate toxicity.

Vitamin preparations containing folic acid or its derivatives may decrease responses to systemically administered methotrexate. Preliminary animal and human studies have

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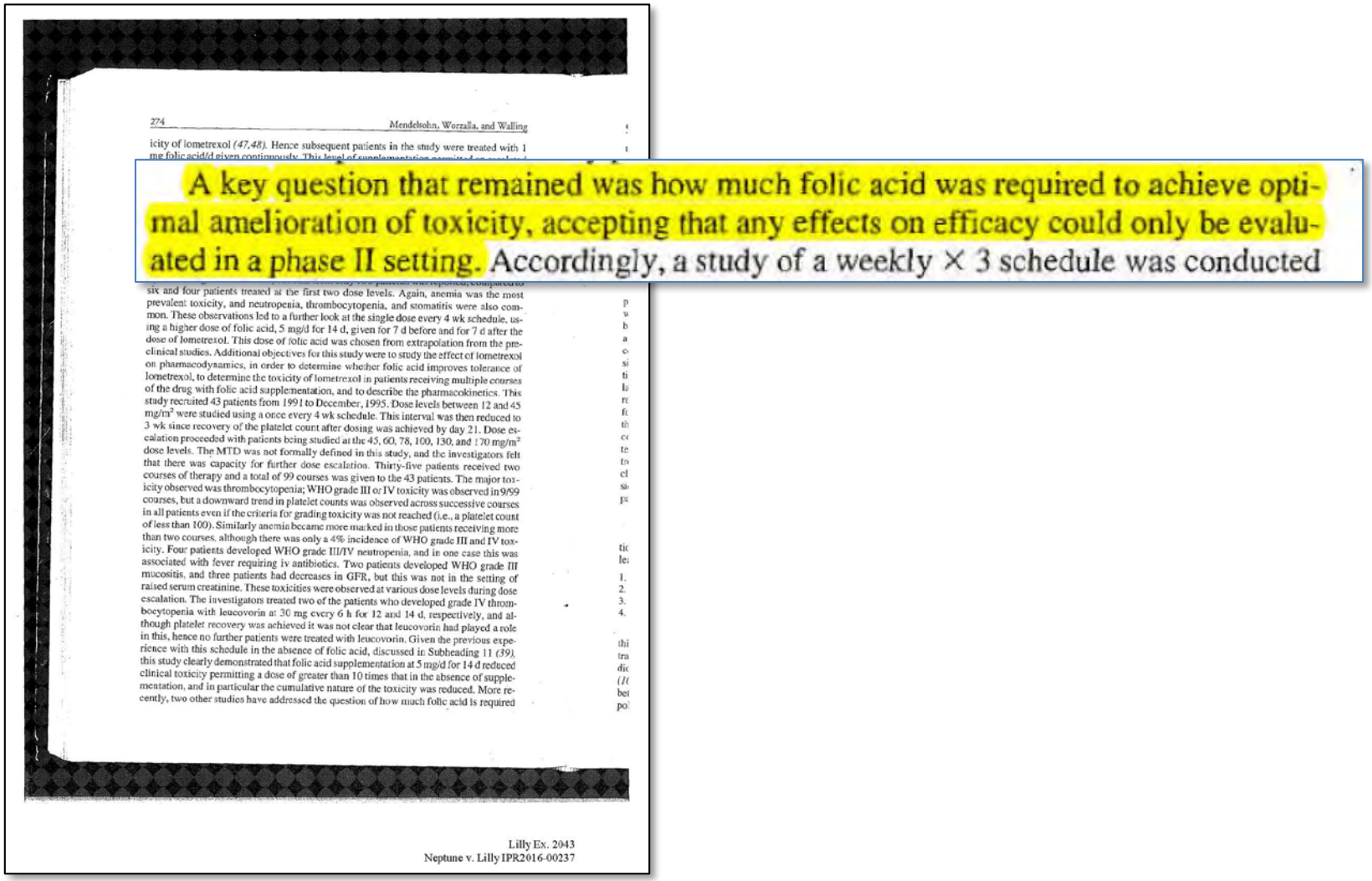
Source: Ex. 2020 at 1398-1399; Paper 48 at 15-16.

State of the Art

Mendelsohn



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icity of lometrexol (47,48). Hence subsequent patients in the study were treated with 1 mg folic acid/d given continuously. This level of supplementation permitted an escalation

six and four patients treated at the first two dose levels. Again, anemia was the most prevalent toxicity, and neutropenia, thrombocytopenia, and stomatitis were also common. These observations led to a further look at the single dose every 4 wk schedule, using a higher dose of folic acid, 5 mg/d for 14 d, given for 7 d before and for 7 d after the dose of lometrexol. This dose of folic acid was chosen from extrapolation from the preclinical studies. Additional objectives for this study were to study the effect of lometrexol on pharmacodynamics, in order to determine whether folic acid improves tolerance of lometrexol, to determine the toxicity of lometrexol in patients receiving multiple courses of the drug with folic acid supplementation, and to describe the pharmacokinetics. This study recruited 43 patients from 1991 to December, 1995. Dose levels between 12 and 45 mg/m^2 were studied using a once every 4 wk schedule. This interval was then reduced to 3 wk since recovery of the platelet count after dosing was achieved by day 21. Dose escalation proceeded with patients being studied at the 45, 60, 78, 100, 130, and 170 mg/m^2 dose levels. The MTD was not formally defined in this study, and the investigators felt that there was capacity for further dose escalation. Thirty-five patients received two courses of therapy and a total of 99 courses was given to the 43 patients. The major toxicity observed was thrombocytopenia; WHO grade III or IV toxicity was observed in 9/99 courses, but a downward trend in platelet counts was observed across successive courses in all patients even if the criteria for grading toxicity was not reached (i.e., a platelet count of less than 100). Similarly anemia became more marked in those patients receiving more than two courses, although there was only a 4% incidence of WHO grade III and IV toxicity. Four patients developed WHO grade III/IV neutropenia, and in one case this was associated with fever requiring iv antibiotics. Two patients developed WHO grade III mucositis, and three patients had decreases in GFR, but this was not in the setting of raised serum creatinine. These toxicities were observed at various dose levels during dose escalation. The investigators treated two of the patients who developed grade IV thrombocytopenia with leucovorin at 30 mg every 6 h for 12 and 14 d, respectively, and although platelet recovery was achieved it was not clear that leucovorin had played a role in this, hence no further patients were treated with leucovorin. Given the previous experience with this schedule in the absence of folic acid, discussed in Subheading 11 (39), this study clearly demonstrated that folic acid supplementation at 5 mg/d for 14 d reduced clinical toxicity permitting a dose of greater than 10 times that in the absence of supplementation, and in particular the cumulative nature of the toxicity was reduced. More recently, two other studies have addressed the question of how much folic acid is required

A key question that remained was how much folic acid was required to achieve optimal amelioration of toxicity, accepting that any effects on efficacy could only be evaluated in a phase II setting. Accordingly, a study of a weekly × 3 schedule was conducted

Lilly Ex. 2043
Neptune v. Lilly IPR2016-00237

Source: Ex. 2043 at 274; Paper 48 at 16.

State of the Art

Laohavinij

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malignant fibrous histiocytoma [9], non-small cell lung

Following the initial clinical evaluation of lometrexol, further studies were performed in mice in an attempt to ameliorate the cumulative toxicity of lometrexol and hence enable repeated courses of the drug to be given. These studies revealed that the therapeutic efficacy and toxicity of lometrexol were highly dependent upon dietary folic acid intake [10, 11] and these preclinical data prompted the Phase I study of lometrexol given with folic acid supplementation described here. The objectives of this clinical Phase I study were:

(a) To evaluate the effect of folic acid on lometrexol pharmacodynamics, in order to determine whether folic acid improves tolerance of lometrexol.

(b) To determine the toxicity of lometrexol in patients receiving multiple courses of the drug with folic acid supplementation.

(c) To describe the pharmacokinetics of lometrexol in patients receiving folic acid supplementation.

Patients and methods

Patient eligibility

From September 1991 to December 1995, 43 patients with a histologically confirmed diagnosis of malignant solid tumour, which was refractory to established therapies or for which no standard therapy existed, were entered into this study. All patients had a predicted life expectancy of at least 12 weeks, and had recovered from the toxicity of previous treatment before entering onto the study. Specifically, patients were required to not have received previous anticancer therapy or other investigational drugs within at least 4 weeks (6 weeks if prior therapy included a nitrosourea, mitomycin C or extensive radiotherapy). Exclusion criteria included factors which could have interfered with lometrexol disposition/toxicity or folic acid absorption, and comprised; (a) concomitant medication with allopurinol, probenecid, nephrotoxic agents, trimethoprim, anti-epileptics, co-trimoxazole or pyrimethamine, (b) extensive radiotherapy and (c) inflammatory ulcerative bowel disease, or malabsorption syndrome. Concurrent treatment with other experimental drugs or other anticancer therapies was not allowed. Patients with clinical evidence or symptoms suggestive of coronary artery disease or central nervous system disease were excluded. Patients with effusions and/or ascites were also not recruited.

All patients were required to have adequate organ function prior to treatment, with marrow function characterised by a white blood cell count of at least 4×10^9/l, neutrophil count at least 2×10^9/l, haemoglobin level of at least 10 g/dl, and platelet count of at least 100×10^9/l. Adequate hepatic function was also required, as characterised by bilirubin levels of < 25 μmol/l, alkaline phosphatase ≤ 2.5 times upper limit of normal, prothrombin and partial thromboplastin time within normal range. The creatinine level was required to be less than 120 μmol/l and the glomerular filtration rate (GFR) to be above 50 ml/min as measured by ^{51}Cr-EDTA clearance.

Study design

Folic acid (Approved Prescription Services Ltd., Leeds, U.K.) was given daily as a single 5 mg tablet for 7 days prior to and 7 days following lometrexol administration at 4 week intervals. Lometrexol (Lilly Research Centre, Erl Wood Manor, Surrey, U.K.) was reconstituted in 0.9% (w/v) saline and administered as a rapid i.v. bolus over 30 seconds to one minute at a concentration of 1–10 mg/ml. Patients were admitted to the Department of Medical Oncology, Newcastle General Hospital, to receive lometrexol and were observed for a further 24 hours following drug administration, to ensure that acute toxicity was not apparent. The following studies were performed weekly: physical examination, toxicity and performance status assessment, and biochemical analysis. Full blood counts were measured twice a week. As part of the Phase I trial of lometrexol with folic acid it was important to demonstrate that plasma folate concentrations of patients were increased by folate supplementation and folate levels were measured on course 1 prior to supplementation (day 7) and after 7 days of folate administration but prior to lometrexol (day 0). Plasma folate concentrations were determined using a commercial folate binding assay (SimulTRAC-SNB, Becton Dickinson, Oxford, UK).

The starting dose was 12 mg/m^2 as this dose of lometrexol given alone had been well tolerated on the first course of therapy in previous Phase I studies, regardless of schedule [5–8]. Lometrexol was given as a single bolus injection every 4 weeks, with 5 mg/day oral folic acid administration 7 days prior to treatment with lometrexol and 7 days afterwards on each course. Toxicities were evaluated according to World Health Organisation (WHO) criteria. If repeated courses at a given dose level were tolerated without toxicity greater

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Following the initial clinical evaluation of lometrexol, further studies were performed in mice in an attempt to ameliorate the cumulative toxicity of lometrexol and hence enable repeated courses of the drug to be given. These studies revealed that the therapeutic efficacy and toxicity of lometrexol were highly dependent upon dietary folic acid intake [10, 11] and these preclinical data prompted the Phase I study of lometrexol given with folic acid supplementation described here. The objectives of this clinical Phase I study were:

(a) To evaluate the effect of folic acid on lometrexol pharmacodynamics, in order to determine whether folic acid improves tolerance of lometrexol.

(b) To determine the toxicity of lometrexol in patients receiving multiple courses of the drug with folic acid supplementation.

(c) To describe the pharmacokinetics of lometrexol in patients receiving folic acid supplementation.

State of the Art

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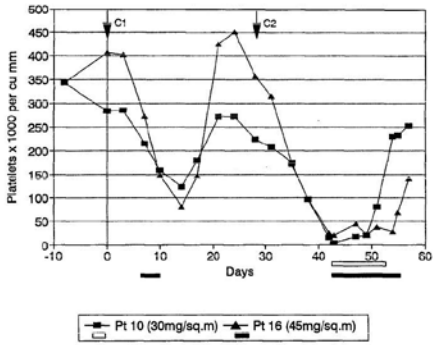

Figure 2. Lometrexol-induced thrombocytopenia in two patients treated with leucovorin. Patients 10 and 16 received 2 courses of lometrexol at 30 and 45 mg/m² every 4 weeks, respectively. The timing of lometrexol administration is represented by arrows. Patient 10 received leucovorin after the second course at the platelet count nadir, represented by the open bar (days 44–55). Patient 16 received leucovorin after the first course (days 7–10), because of grade III diarrhoea, and after the second course at the platelet count nadir (days 43–56), represented by the solid bars.

change in lung lesions on chest x-ray). Four patients (1 metastatic malignant melanome, 1 ovarian cancer and 2 unknown primary carcinoma) had disease stabilization for 4, 3, 3 and 5 months, as assessed by physical examination and computed tomography scan.

Plasma folate status following oral folic acid supplementation

Plasma folate concentrations were measured in 23 patients prior to folate supplementation (day –7) and after 7 days of 5 mg/day oral folic acid, i.e. prior to lometrexol administration (day 0). Plasma folate concentrations were significantly increased from 9 (3–63) to 20 (6–180) ng/ml [median (range)] after 7 days of folate supplementation (paired *t*-test $p = 0.009$). Pre and post folate supplementation plasma folate concentrations were variable, increasing in most but not all cases following the first week of folate supplementation by 10 (–6 to 134) ng/ml [median (range)], amounting to a 100% (–20% to 1400%) change [median (range)].

Pharmacokinetics

For the dose range 12–45 mg/m², as previously described [12], there was a linear relationship between lometrexol dose and area under the plasma concentration *versus* time curve. In the present study, this relationship was maintained at doses up to and including 130 mg/m² ($r^2 = 0.89$).

Discussion

The objective of the present clinical study was to identify a safe dose of lometrexol when given with folate supplementation so as to allow Phase II trials, in an attempt to reproduce the efficacy of lometrexol seen in folate-deficient mice receiving folate supplementation. The most common and severe toxicities in this study were thrombocytopenia and mucositis. Leucopenia, neutropenia, anaemia and diarrhoea were mild and infrequent. Reductions of GFR of 21–50% and > 50% after treatment with lometrexol were also observed in 3

The objective of the present clinical study was to identify a safe dose of lometrexol when given with folate supplementation so as to allow Phase II trials, in an attempt to reproduce the efficacy of lometrexol seen in folate-deficient mice receiving folate supplementation. The most common and severe toxicities in this

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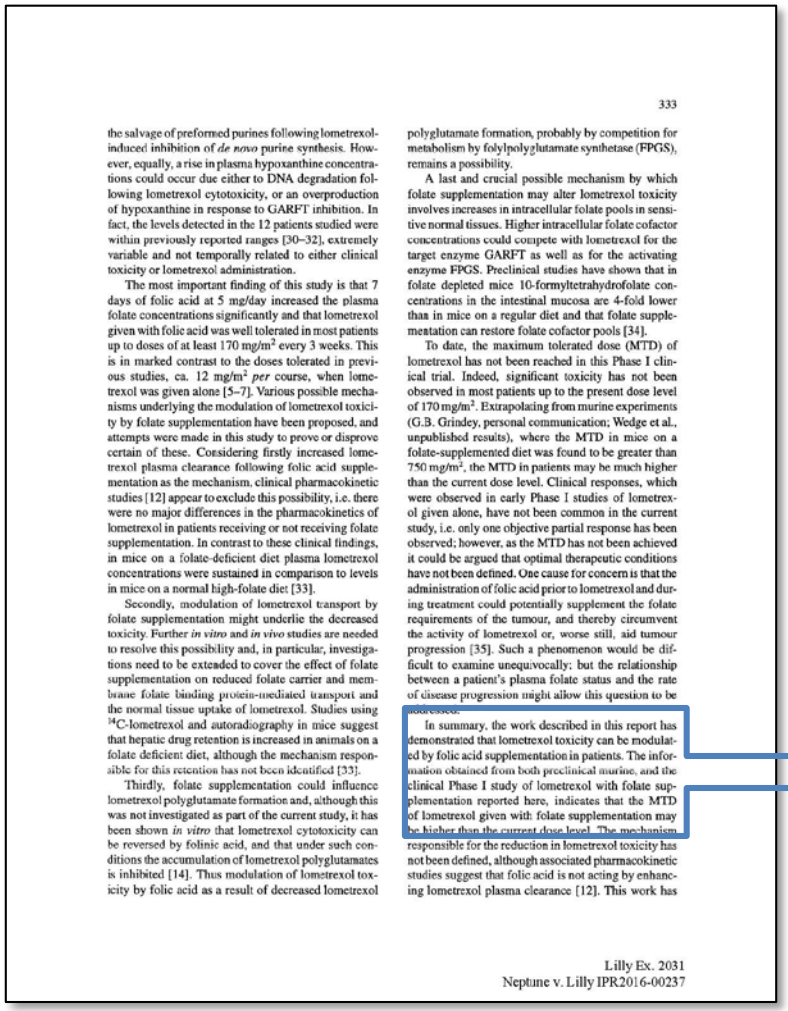

the salvage of preformed purines following lometrexol-induced inhibition of *de novo* purine synthesis. However, equally, a rise in plasma hypoxanthine concentrations could occur due either to DNA degradation following lometrexol cytotoxicity, or an overproduction of hypoxanthine in response to GARFT inhibition. In fact, the levels detected in the 12 patients studied were within previously reported ranges [30–32], extremely variable and not temporally related to either clinical toxicity or lometrexol administration.

The most important finding of this study is that 7 days of folic acid at 5 mg/day increased the plasma folate concentrations significantly and that lometrexol given with folic acid was well tolerated in most patients up to doses of at least 170 mg/m^2 every 3 weeks. This is in marked contrast to the doses tolerated in previous studies, ca. 12 mg/m^2 *per* course, when lometrexol was given alone [5–7]. Various possible mechanisms underlying the modulation of lometrexol toxicity by folate supplementation have been proposed, and attempts were made in this study to prove or disprove certain of these. Considering firstly increased lometrexol plasma clearance following folic acid supplementation as the mechanism, clinical pharmacokinetic studies [12] appear to exclude this possibility, i.e. there were no major differences in the pharmacokinetics of lometrexol in patients receiving or not receiving folate supplementation. In contrast to these clinical findings, in mice on a folate-deficient diet plasma lometrexol concentrations were sustained in comparison to levels in mice on a normal high-folate diet [33].

Secondly, modulation of lometrexol transport by folate supplementation might underlie the decreased toxicity. Further *in vitro* and *in vivo* studies are needed to resolve this possibility and, in particular, investigations need to be extended to cover the effect of folate supplementation on reduced folate carrier and membrane folate binding protein-mediated transport and the normal tissue uptake of lometrexol. Studies using ^{14}C-lometrexol and autoradiography in mice suggest that hepatic drug retention is increased in animals on a folate deficient diet, although the mechanism responsible for this retention has not been identified [33].

Thirdly, folate supplementation could influence lometrexol polyglutamate formation and, although this was not investigated as part of the current study, it has been shown *in vitro* that lometrexol cytotoxicity can be reversed by folinic acid, and that under such conditions the accumulation of lometrexol polyglutamates is inhibited [14]. Thus modulation of lometrexol toxicity by folic acid as a result of decreased lometrexol polyglutamate formation, probably by competition for metabolism by folylpolyglutamate synthetase (FPGS), remains a possibility.

A last and crucial possible mechanism by which folate supplementation may alter lometrexol toxicity involves increases in intracellular folate pools in sensitive normal tissues. Higher intracellular folate cofactor concentrations could compete with lometrexol for the target enzyme GARFT as well as for the activating enzyme FPGS. Preclinical studies have shown that in folate depleted mice 10-formyltetrahydrofolate concentrations in the intestinal mucosa are 4-fold lower than in mice on a regular diet and that folate supplementation can restore folate cofactor pools [34].

To date, the maximum tolerated dose (MTD) of lometrexol has not been reached in this Phase I clinical trial. Indeed, significant toxicity has not been observed in most patients up to the present dose level of 170 mg/m^2. Extrapolating from murine experiments (G.B. Grindey, personal communication; Wedge et al., unpublished results), where the MTD in mice on a folate-supplemented diet was found to be greater than 750 mg/m^2, the MTD in patients may be much higher than the current dose level. Clinical responses, which were observed in early Phase I studies of lometrexol given alone, have not been common in the current study, i.e. only one objective partial response has been observed; however, as the MTD has not been achieved it could be argued that optimal therapeutic conditions have not been defined. One cause for concern is that the administration of folic acid prior to lometrexol and during treatment could potentially supplement the folate requirements of the tumour, and thereby circumvent the activity of lometrexol or, worse still, aid tumour progression [35]. Such a phenomenon would be difficult to examine unequivocally; but the relationship between a patient's plasma folate status and the rate of disease progression might allow this question to be addressed.

In summary, the work described in this report has demonstrated that lometrexol toxicity can be modulated by folic acid supplementation in patients. The information obtained from both preclinical murine, and the clinical Phase I study of lometrexol with folate supplementation reported here, indicates that the MTD of lometrexol given with folate supplementation may be higher than the current dose level. The mechanism responsible for the reduction in lometrexol toxicity has not been defined, although associated pharmacokinetic studies suggest that folic acid is not acting by enhancing lometrexol plasma clearance [12]. This work has

In summary, the work described in this report has demonstrated that lometrexol toxicity can be modulated by folic acid supplementation in patients. The information obtained from both preclinical murine, and the clinical Phase I study of lometrexol with folate supplementation reported here, indicates that the MTD of lometrexol given with folate supplementation may be higher than the current dose level. The mechanism

Source: Ex. 2031 at 333; Paper 48 at 17.

State of the Art

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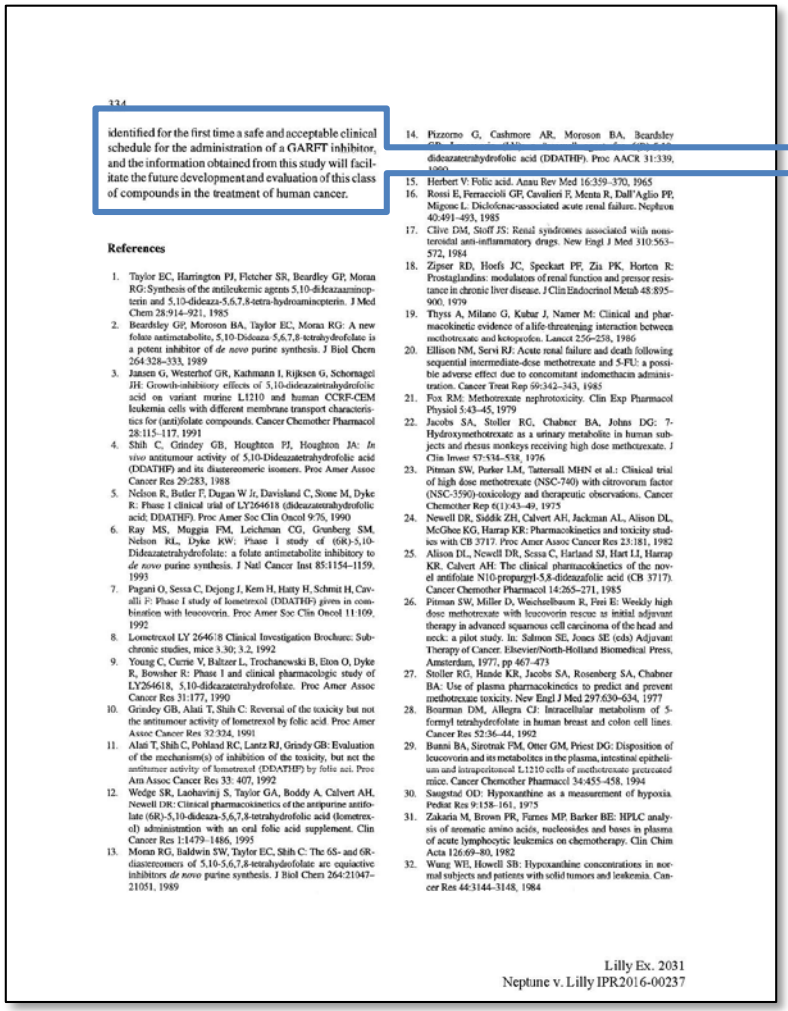

identified for the first time a safe and acceptable clinical schedule for the administration of a GARFT inhibitor, and the information obtained from this study will facilitate the future development and evaluation of this class of compounds in the treatment of human cancer.

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Source: Ex. 2031 at 334; Paper 48 at 17.

State of the Art

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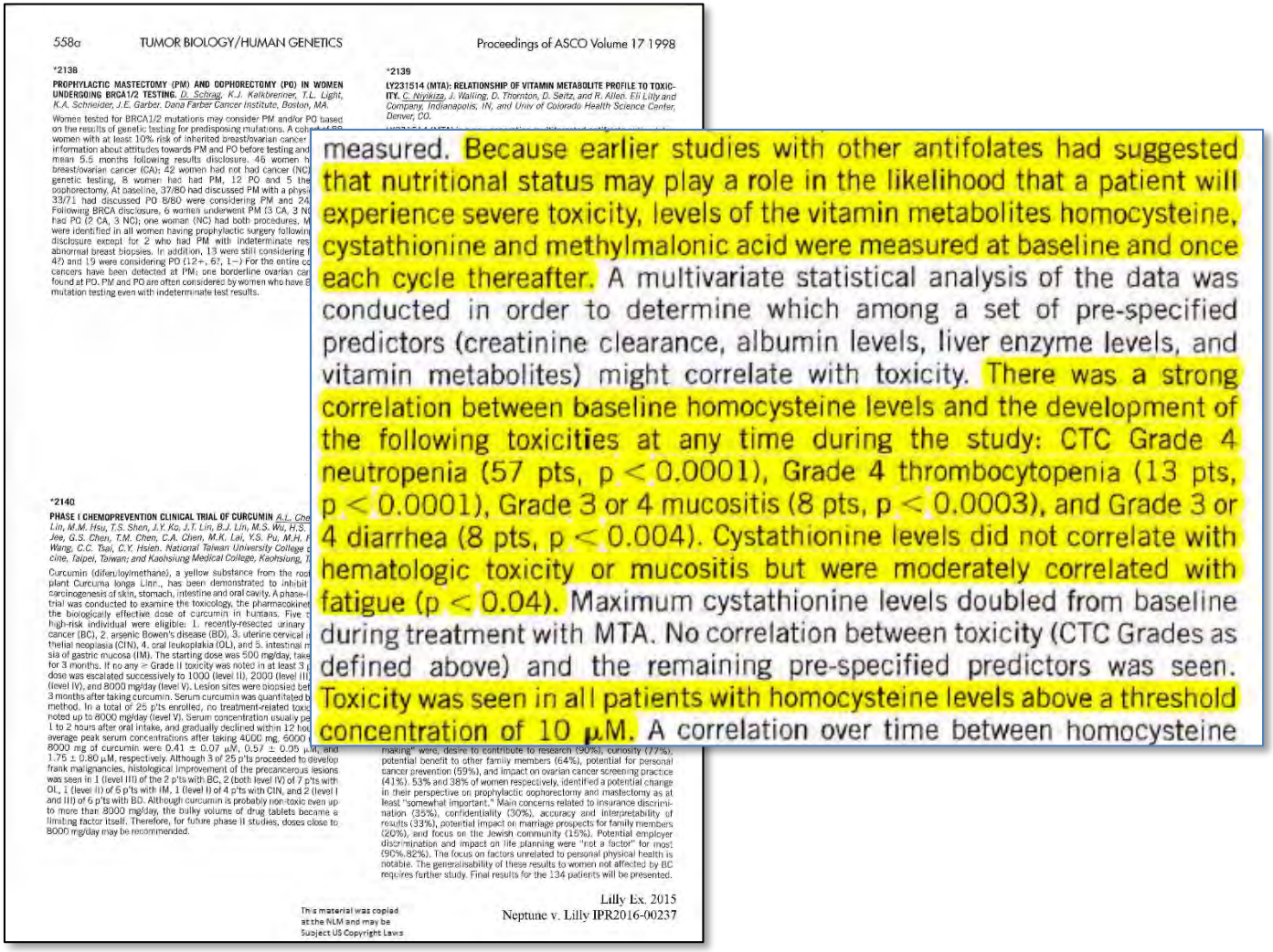

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PROPHYLACTIC MASTECTOMY (PM) AND OOPHORECTOMY (PO) IN WOMEN UNDERGOING BRCA1/2 TESTING. *D. Schrag, K.J. Kalkbrenner, T.L. Light, K.A. Schneider, J.E. Garber, Dana Farber Cancer Institute, Boston, MA.*

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 information about attitudes towards PM and PO before testing and mean 5.5 months following results disclosure. 46 women h breast/ovarian cancer (CA); 42 women had not had cancer (NC) genetic testing, 8 women had had PM, 12 PO and 5 the oophorectomy. At baseline, 37/80 had discussed PM with a physi 33/71 had discussed PO 8/80 were considering PM and 24/ Following BRCA disclosure, 6 women underwent PM (3 CA, 3 NC had PO (2 CA, 3 NC); one woman (NC) had both procedures. M were identified in all women having prophylactic surgery followin disclosure except for 2 who had PM with indeterminate res abnormal breast biopsies. In addition, 13 were still considering f 4/) and 19 were considering PO (12+, 6?, 1–) For the entire co cancers have been detected at PM; one borderline ovarian can found at PO. PM and PO are often considered by women who have B 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.*

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

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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PHASE I CHEMOPREVENTION CLINICAL TRIAL OF CURCUMIN *A.L. Cheng, C.H. Lin, M.M. Hsu, T.S. Shen, J.Y. Ko, J.T. Lin, B.J. Lin, M.S. Wu, H.S. Yu, S. 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 plant Curcuma longa Linn., has been demonstrated to inhibit carcinogenesis of skin, stomach, intestine and oral cavity. A phase-I trial was conducted to examine the toxicology, the pharmacokinetics, the biologically effective dose of curcumin in humans. Five types high-risk individual were eligible: 1. recently-resected urinary 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 for 3 months. If no any ≥ Grade II toxicity was noted in at least 3 pts, 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.

Source: Ex. 2015 at 558a; Paper 48 at 18-20.

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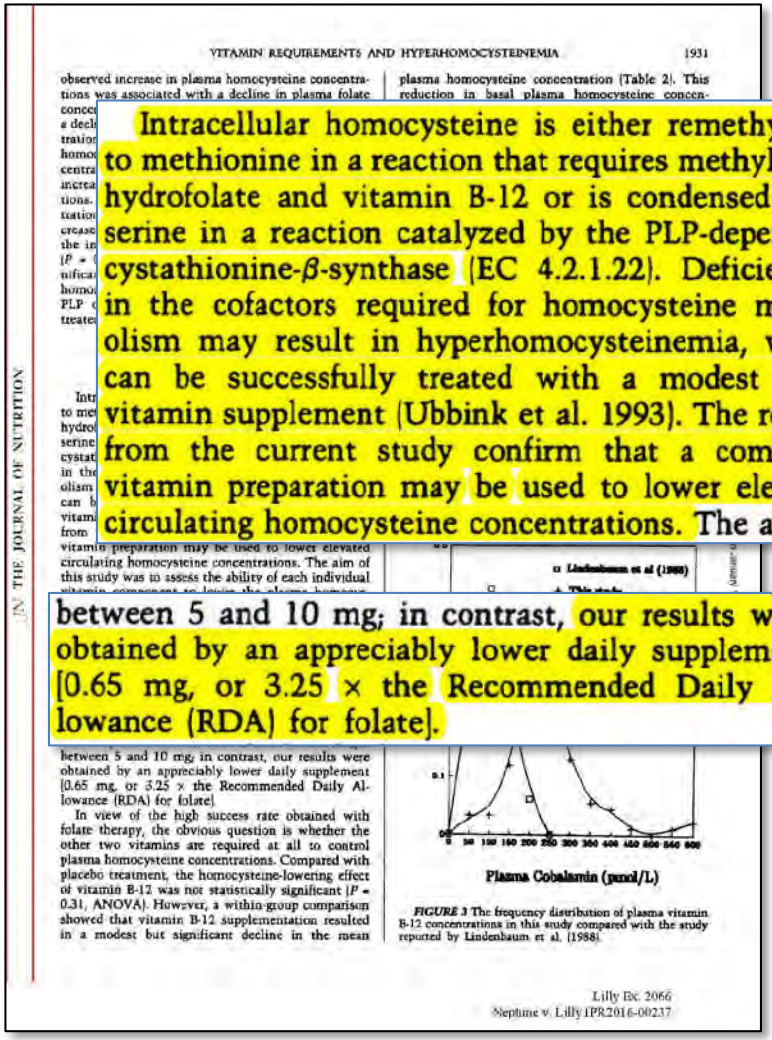

VITAMIN REQUIREMENTS AND HYPERHOMOCYSTEINEMIA 1931

observed increase in plasma homocysteine concentrations was associated with a decline in plasma folate

plasma homocysteine concentration (Table 2). This reduction in basal plasma homocysteine concen-

Intracellular homocysteine is either remethylated to methionine in a reaction that requires methyltetrahydrofolate and vitamin B-12 or is condensed with serine in a reaction catalyzed by the PLP-dependent cystathionine-β-synthase (EC 4.2.1.22). Deficiencies in the cofactors required for homocysteine metabolism may result in hyperhomocysteinemia, which can be successfully treated with a modest daily vitamin supplement (Ubbink et al. 1993). The results from the current study confirm that a combined vitamin preparation may be used to lower elevated circulating homocysteine concentrations. The aim of

showed that vitamin B-12 supplementation resulted in a modest but significant decline in the mean plasma homocysteine concentration (Table 2). This

min, whereas in our study the hyperhomocysteinemic men were randomized into the different treatment groups without prior knowledge of vitamin nutritional status or any possible genetic aberrations. The

between 5 and 10 mg; in contrast, our results were obtained by an appreciably lower daily supplement [0.65 mg, or 3.25 × the Recommended Daily Allowance (RDA) for folate].

between 5 and 10 mg; in contrast, our results were obtained by an appreciably lower daily supplement [0.65 mg, or 3.25 × the Recommended Daily Allowance (RDA) for folate].

In view of the high success rate obtained with folate therapy, the obvious question is whether the other two vitamins are required at all to control plasma homocysteine concentrations. Compared with placebo treatment, the homocysteine-lowering effect of vitamin B-12 was not statistically significant [$P = 0.31$, ANOVA]. However, a within-group comparison showed that vitamin B-12 supplementation resulted in a modest but significant decline in the mean

Plasma Cobalamin (pmol/L)

FIGURE 3 The frequency distribution of plasma vitamin B-12 concentrations in this study compared with the study reported by Lindenbaum et al. (1988).

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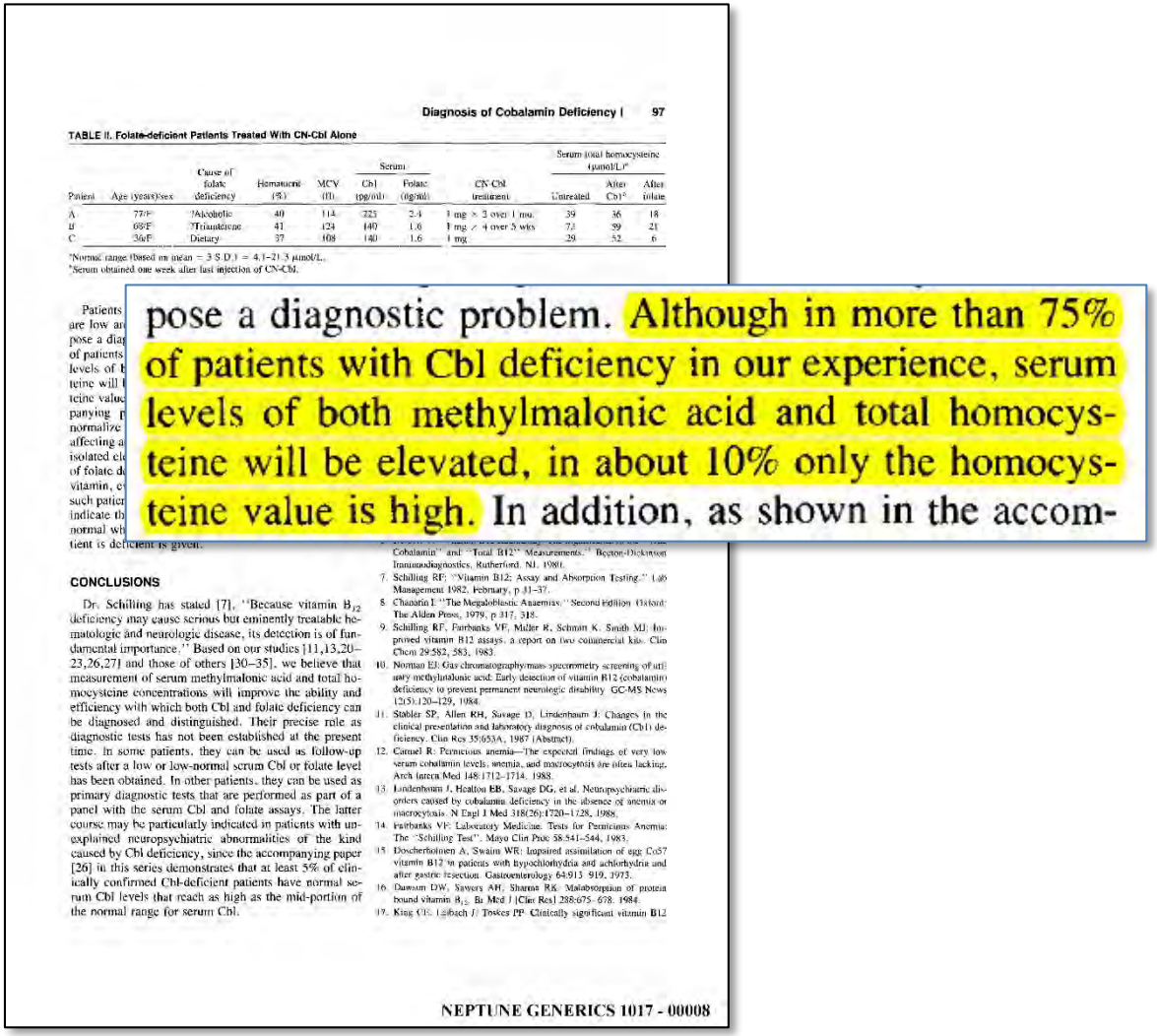
Folic acid supplementation in patients with a chronic vitamin B-12 deficiency may eventually result in neuropathy due to failure to recognize the vitamin B-12 deficiency (Beck 1991). Moreover, Allen et al. (1990) have recently shown that folate supplementation will not correct hyperhomocysteinemia that is primarily the result of a vitamin B-12 deficiency. It is therefore essential that vitamin B-12 and folate be combined to treat hyperhomocysteinemia.

Source: Ex. 2066 at 1931; Paper 48 at 20.

State of the Art

Allen



Diagnosis of Cobalamin Deficiency I 97

TABLE II. Folate-deficient Patients Treated With CN-Cbl Alone

Patient	Age (years)/sex	Cause of folate deficiency	Hematocrit (%)	MCV (fl)	Serum Cbl (pg/ml)	Serum Folate (ng/ml)	CN-Cbl treatment	Serum total homocysteine (μmol/L)* Untreated	After Cbl†	After folate
A	77/F	Alcoholic	40	114	225	2.4	1 mg × 2 over 1 mo.	39	36	18
B	68/F	Triamterene	41	124	140	1.6	1 mg × 4 over 5 wks	73	59	21
C	36/F	Dietary	37	108	140	1.6	1 mg	29	52	6

*Normal range (based on mean ± 3 S.D.) = 4.1–21.3 μmol/L.
†Serum obtained one week after last injection of CN-Cbl.

pose a diagnostic problem. Although in more than 75% of patients with Cbl deficiency in our experience, serum levels of both methylmalonic acid and total homocysteine will be elevated, in about 10% only the homocysteine value is high. In addition, as shown in the accom-

CONCLUSIONS

Dr. Schilling has stated [7], "Because vitamin B_{12} deficiency may cause serious but eminently treatable hematologic and neurologic disease, its detection is of fundamental importance." Based on our studies [11,13,20–23,26,27] and those of others [30–35], we believe that measurement of serum methylmalonic acid and total homocysteine concentrations will improve the ability and efficiency with which both Cbl and folate deficiency can be diagnosed and distinguished. Their precise role as diagnostic tests has not been established at the present time. In some patients, they can be used as follow-up tests after a low or low-normal serum Cbl or folate level has been obtained. In other patients, they can be used as primary diagnostic tests that are performed as part of a panel with the serum Cbl and folate assays. The latter course may be particularly indicated in patients with unexplained neuropsychiatric abnormalities of the kind caused by Cbl deficiency, since the accompanying paper [26] in this series demonstrates that at least 5% of clinically confirmed Cbl-deficient patients have normal serum Cbl levels that reach as high as the mid-portion of the normal range for serum Cbl.

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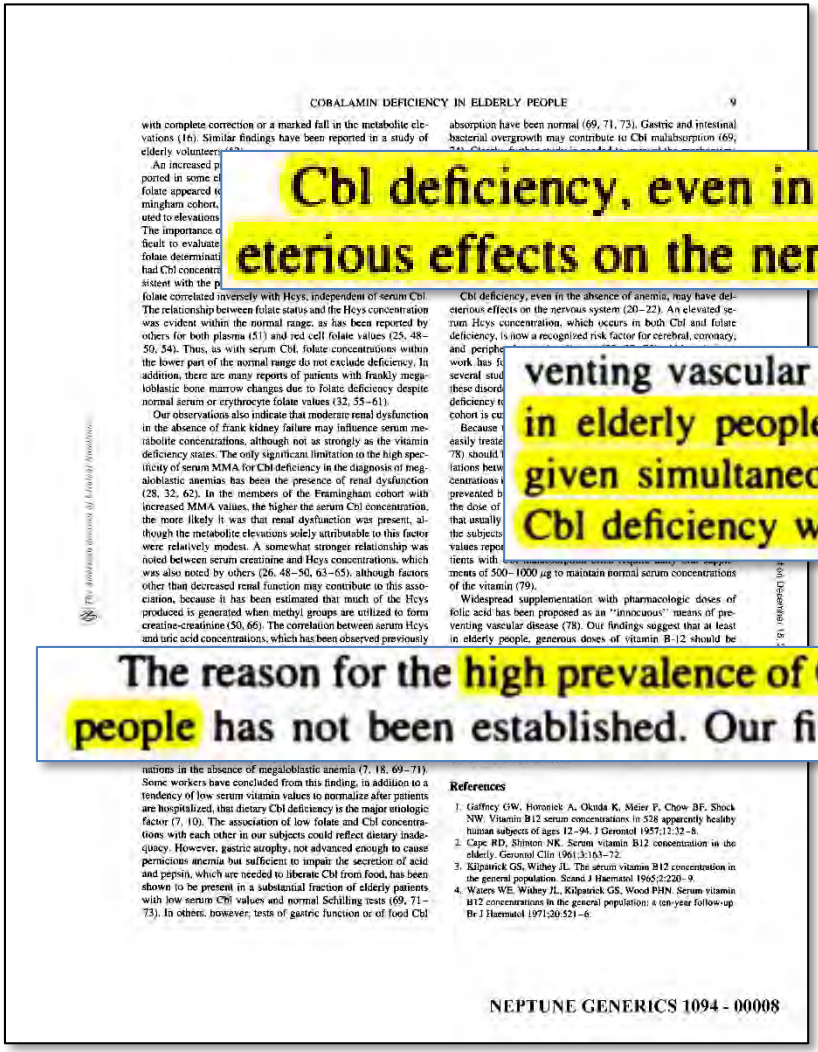

COBALAMIN DEFICIENCY IN ELDERLY PEOPLE 9

with complete correction or a marked fall in the metabolite elevations (16). Similar findings have been reported in a study of elderly volunteers

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folate correlated inversely with Heys, independent of serum Cbl. The relationship between folate status and the Heys concentration was evident within the normal range, as has been reported by others for both plasma (51) and red cell folate values (25, 48–50, 54). Thus, as with serum Cbl, folate concentrations within the lower part of the normal range do not exclude deficiency. In addition, there are many reports of patients with frankly megaloblastic bone marrow changes due to folate deficiency despite normal serum or erythrocyte folate values (32, 55–61).

Our observations also indicate that moderate renal dysfunction in the absence of frank kidney failure may influence serum metabolite concentrations, although not as strongly as the vitamin deficiency states. The only significant limitation to the high specificity of serum MMA for Cbl deficiency in the diagnosis of megaloblastic anemias has been the presence of renal dysfunction (28, 32, 62). In the members of the Framingham cohort with increased MMA values, the higher the serum Cbl concentration, the more likely it was that renal dysfunction was present, although the metabolite elevations solely attributable to this factor were relatively modest. A somewhat stronger relationship was noted between serum creatinine and Heys concentrations, which was also noted by others (26, 48–50, 63–65), although factors other than decreased renal function may contribute to this association, because it has been estimated that much of the Heys produced is generated when methyl groups are utilized to form creatine-creatinine (50, 66). The correlation between serum Heys and uric acid concentrations, which has been observed previously

nations in the absence of megaloblastic anemia (7, 18, 69–71). Some workers have concluded from this finding, in addition to a tendency of low serum vitamin values to normalize after patients are hospitalized, that dietary Cbl deficiency is the major etiologic factor (7, 10). The association of low folate and Cbl concentrations with each other in our subjects could reflect dietary inadequacy. However, gastric atrophy, not advanced enough to cause pernicious anemia but sufficient to impair the secretion of acid and pepsin, which are needed to liberate Cbl from food, has been shown to be present in a substantial fraction of elderly patients with low serum Cbl values and normal Schilling tests (69, 71–73). In others, however, tests of gastric function or of food Cbl absorption have been normal (69, 71, 73). Gastric and intestinal bacterial overgrowth may contribute to Cbl malabsorption (69,

Cbl deficiency, even in the absence of anemia, may have deleterious effects on the nervous system (20–22). An elevated serum Heys concentration, which occurs in both Cbl and folate deficiency, is now a recognized risk factor for cerebral, coronary,

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Cbl deficiency, even in the absence of anemia, may have deleterious effects on the nervous system (20–22). An elevated se-

venting vascular disease (78). Our findings suggest that at least in elderly people, generous doses of vitamin B-12 should be given simultaneously to prevent inappropriate mistreatment of Cbl deficiency with folic acid. The effects of such supplemen-

The reason for the high prevalence of Cbl deficiency in elderly people has not been established. Our finding that only 1 of 67

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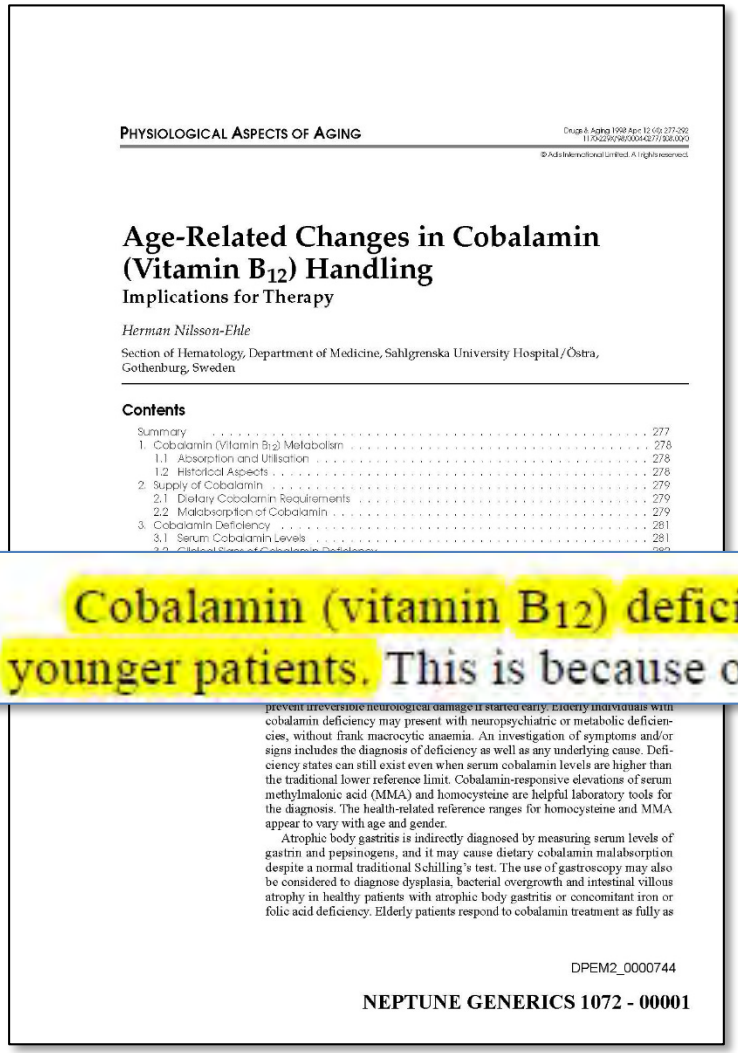
Nilsson-Ehle



PHYSIOLOGICAL ASPECTS OF AGING

Age-Related Changes in Cobalamin (Vitamin B_{12}) Handling

Implications for Therapy

Herman Nilsson-Ehle

Section of Hematology, Department of Medicine, Sahlgrenska University Hospital/Östra, Gothenburg, Sweden

Contents

Cobalamin (vitamin B_{12}) deficiency is more common in the elderly than in younger patients. This is because of the increased prevalence of cobalamin mal-

prevent irreversible neurological damage if started early. Elderly individuals with cobalamin deficiency may present with neuropsychiatric or metabolic deficiencies, without frank macrocytic anaemia. An investigation of symptoms and/or signs includes the diagnosis of deficiency as well as any underlying cause. Deficiency states can still exist even when serum cobalamin levels are higher than the traditional lower reference limit. Cobalamin-responsive elevations of serum methylmalonic acid (MMA) and homocysteine are helpful laboratory tools for the diagnosis. The health-related reference ranges for homocysteine and MMA appear to vary with age and gender.

Atrophic body gastritis is indirectly diagnosed by measuring serum levels of gastrin and pepsinogens, and it may cause dietary cobalamin malabsorption despite a normal traditional Schilling's test. The use of gastroscopy may also be considered to diagnose dysplasia, bacterial overgrowth and intestinal villous atrophy in healthy patients with atrophic body gastritis or concomitant iron or folic acid deficiency. Elderly patients respond to cobalamin treatment as fully as

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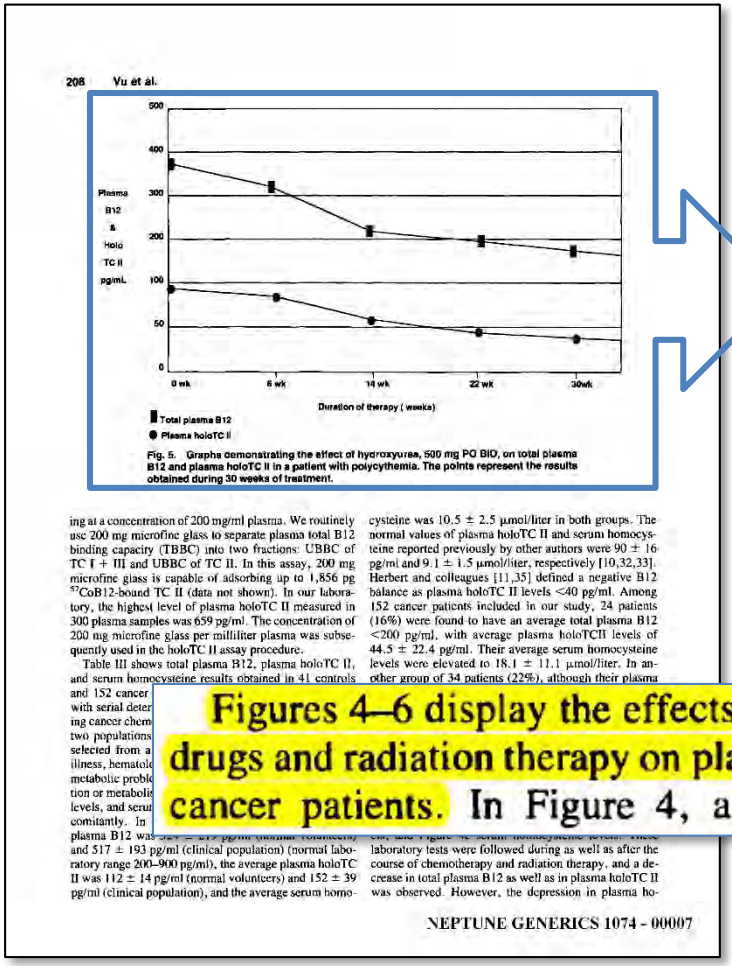

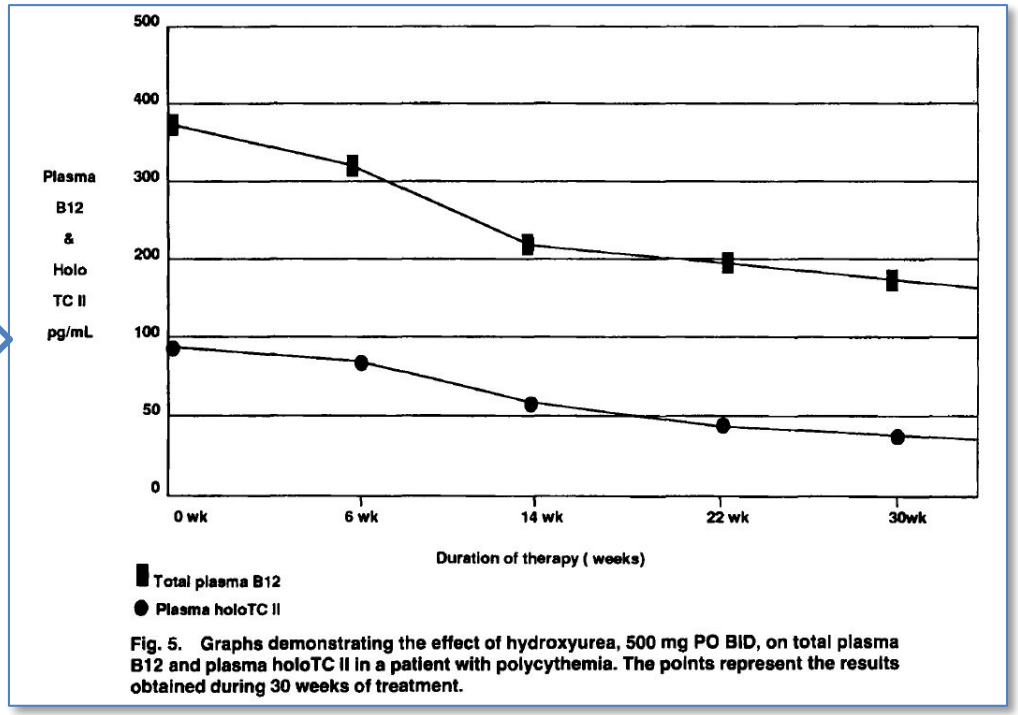

Figures 4–6 display the effects of some chemotherapy drugs and radiation therapy on plasma holoTC II levels in cancer patients. In Figure 4, a patient received three

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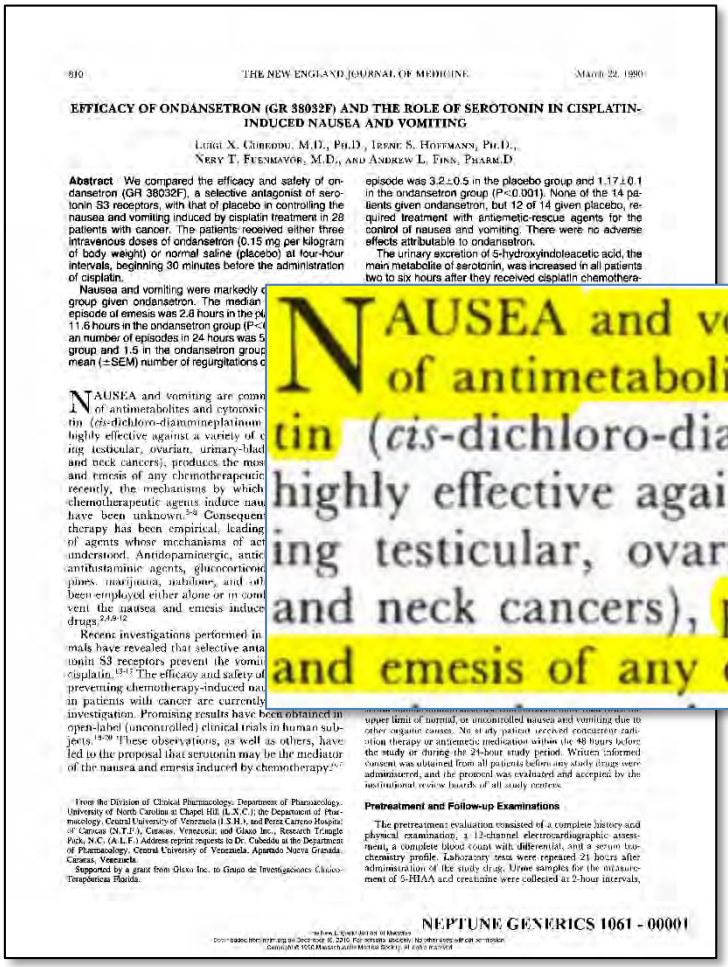

810 THE NEW ENGLAND JOURNAL OF MEDICINE March 22, 1990

EFFICACY OF ONDANSETRON (GR 38032F) AND THE ROLE OF SEROTONIN IN CISPLATIN-INDUCED NAUSEA AND VOMITING

LUIGI X. CUBEDDU, M.D., PH.D., IRENE S. HOFFMANN, PH.D., NERY T. FUENMAYOR, M.D., AND ANDREW L. FINN, PHARM.D.

Abstract We compared the efficacy and safety of ondansetron (GR 38032F), a selective antagonist of serotonin S3 receptors, with that of placebo in controlling the nausea and vomiting induced by cisplatin treatment in 28 patients with cancer. The patients received either three intravenous doses of ondansetron (0.15 mg per kilogram of body weight) or normal saline (placebo) at four-hour intervals, beginning 30 minutes before the administration of cisplatin.

Nausea and vomiting were markedly d... group given ondansetron. The median ... episode of emesis was 2.8 hours in the pl... 11.6 hours in the ondansetron group (P<... an number of episodes in 24 hours was 5... group and 1.5 in the ondansetron group... mean (±SEM) number of regurgitations ...

episode was 3.2±0.5 in the placebo group and 1.17±0.1 in the ondansetron group (P<0.001). None of the 14 patients given ondansetron, but 12 of 14 given placebo, required treatment with antiemetic-rescue agents for the control of nausea and vomiting. There were no adverse effects attributable to ondansetron.

The urinary excretion of 5-hydroxyindoleacetic acid, the main metabolite of serotonin, was increased in all patients two to six hours after they received cisplatin chemothera-

NAUSEA and vomiting are common side effects of antimetabolites and cytotoxic drugs.[1] Cisplatin (*cis*-dichloro-diammineplatinum II), an agent highly effective against a variety of cancers (including testicular, ovarian, urinary-bladder, and head and neck cancers), produces the most severe nausea and emesis of any chemotherapeutic agent.[2-4] Until

...ausea and vomiting are comm... ...of antimetabolites and cytotoxic... ...tin (*cis*-dichloro-diammineplatinum... ...highly effective against a variety of c... ...ing testicular, ovarian, urinary-blad... ...and neck cancers), produces the mos... ...and emesis of any chemotherapeutic... ...recently, the mechanisms by which... ...chemotherapeutic agents induce nau... ...have been unknown.[5-8] Consequen... ...therapy has been empirical, leading... ...of agents whose mechanisms of act... ...understood. Antidopaminergic, antic... ...antihistaminic agents, glucocorticoi... ...pines, marijuana, nabilone, and [illegible] ...been employed either alone or in com... ...vent the nausea and emesis induce... ...drugs.[2,9-12]

Recent investigations performed in ... ...mals have revealed that selective anta... ...tonin S3 receptors prevent the vomi... ...cisplatin.[13-17] The efficacy and safety of ... ...preventing chemotherapy-induced nau... ...in patients with cancer are currently ... ...investigation. Promising results have been obtained in ... ...open-label (uncontrolled) clinical trials in human sub... ...jects.[18-20] These observations, as well as others, have led to the proposal that serotonin may be the mediator of the nausea and emesis induced by chemotherapy.[5-7]

From the Division of Clinical Pharmacology, Department of Pharmacology, University of North Carolina at Chapel Hill (L.X.C.); the Department of Pharmacology, Central University of Venezuela (I.S.H.), and Perez Carreno Hospital of Caracas (N.T.F.), Caracas, Venezuela; and Glaxo Inc., Research Triangle Park, N.C. (A.L.F.) Address reprint requests to Dr. Cubeddu at the Department of Pharmacology, Central University of Venezuela, Apartado Nueva Granada, Caracas, Venezuela.

Supported by a grant from Glaxo Inc. to Grupo de Investigaciones Clinico-Terapeuticas Florida.

upper limit of normal, or uncontrolled nausea and vomiting due to other organic causes. No study patient received concurrent cardiotoxic therapy or antiemetic medication within the 48 hours before the study or during the 24-hour study period. Written informed consent was obtained from all patients before any study drugs were administered, and the protocol was evaluated and accepted by the institutional review boards of all study centers.

Pretreatment and Follow-up Examinations

The pretreatment evaluation consisted of a complete history and physical examination, a 12-channel electrocardiographic assessment, a complete blood count with differential, and a serum biochemistry profile. Laboratory tests were repeated 21 hours after administration of the study drug. Urine samples for the measurement of 5-HIAA and creatinine were collected in 2-hour intervals,

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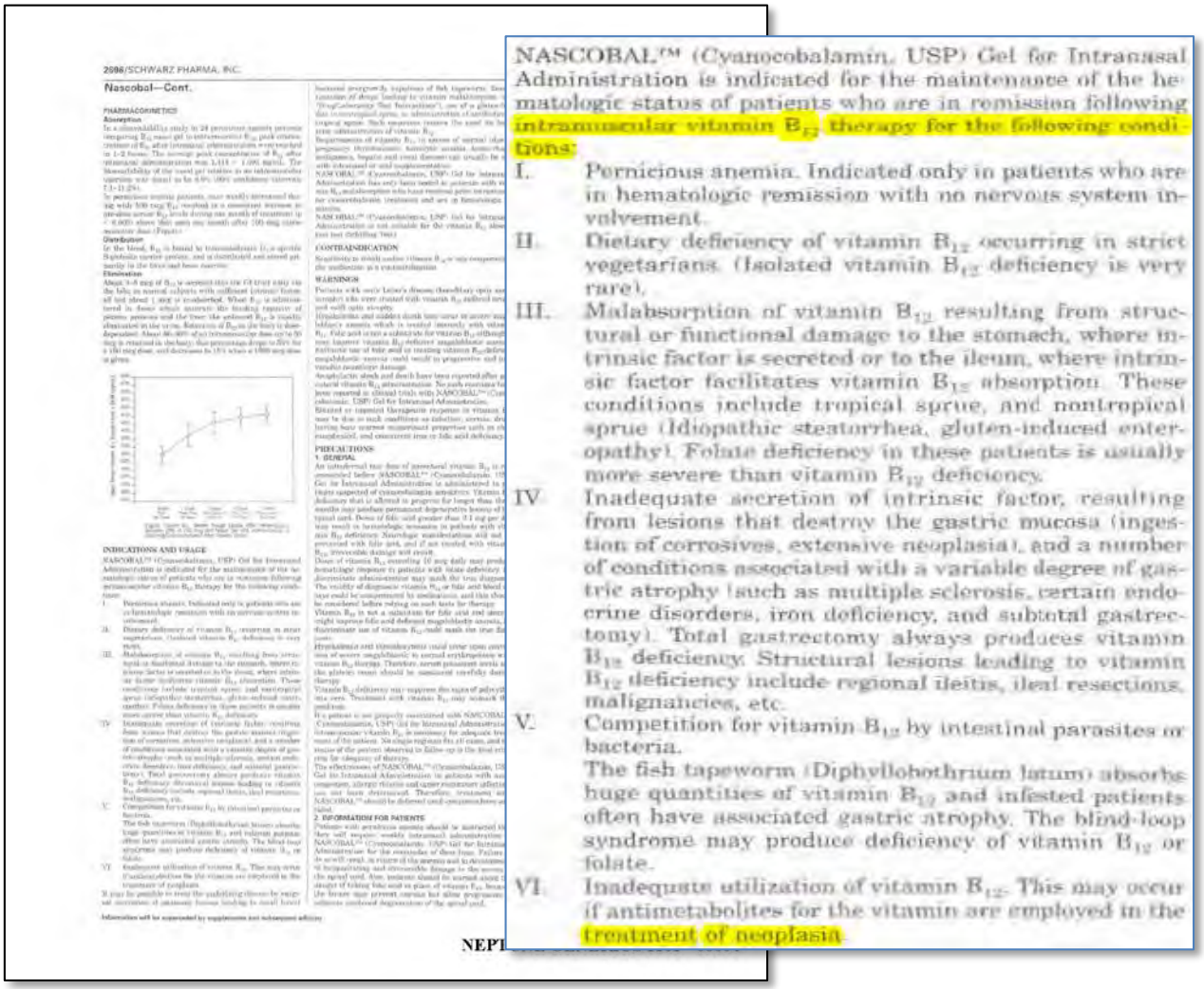

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Nascobal—Cont.

NASCOBAL™ (Cyanocobalamin, USP) Gel for Intranasal Administration is indicated for the maintenance of the hematologic status of patients who are in remission following intramuscular vitamin B_{12} therapy for the following conditions:

I. Pernicious anemia. Indicated only in patients who are in hematologic remission with no nervous system involvement.

II. Dietary deficiency of vitamin B_{12} occurring in strict vegetarians. (Isolated vitamin B_{12} deficiency is very rare).

III. Malabsorption of vitamin B_{12} resulting from structural or functional damage to the stomach, where intrinsic factor is secreted or to the ileum, where intrinsic factor facilitates vitamin B_{12} absorption. These conditions include tropical sprue, and nontropical sprue (Idiopathic steatorrhea, gluten-induced enteropathy). Folate deficiency in these patients is usually more severe than vitamin B_{12} deficiency.

IV. Inadequate secretion of intrinsic factor, resulting from lesions that destroy the gastric mucosa (ingestion of corrosives, extensive neoplasia), and a number of conditions associated with a variable degree of gastric atrophy (such as multiple sclerosis, certain endocrine disorders, iron deficiency, and subtotal gastrectomy). Total gastrectomy always produces vitamin B_{12} deficiency. Structural lesions leading to vitamin B_{12} deficiency include regional ileitis, ileal resections, malignancies, etc.

V. Competition for vitamin B_{12} by intestinal parasites or bacteria.
The fish tapeworm (Diphyllobothrium latum) absorbs huge quantities of vitamin B_{12} and infested patients often have associated gastric atrophy. The blind-loop syndrome may produce deficiency of vitamin B_{12} or folate.

VI. Inadequate utilization of vitamin B_{12}. This may occur if antimetabolites for the vitamin are employed in the treatment of neoplasia.

State of the Art

McLean

Cobalamin Analogues Modulate the Growth of Leukemia Cells *in Vitro*[1]

The Biomedical Research Centre (C. R. M., J. W. S., H. J. Z.) and Departments of Medicine (J. W. S.) and Pathology and Laboratory Medicine (H. J. Z.), University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3; Department of Radiation Oncology, University of Washington, Seattle, WA 98103 (P. M. P., D. S. W.); and Receptagen Corp., Edmonds, WA 98020 (A. C. M., C. S. W.)

ABSTRACT

Analogues of cyanocobalamin (CN-Cbl), with functional groups attached to either the various propionamide groups of the corrin ring or to the ribose-nucleotide linker arm, have been evaluated in a cobalamin (Cbl)-dependent *in vitro* cell growth assay. In this bioassay, CN-Cbl supported, in a dose-dependent manner, the growth of the murine lymphoma BW5147 and the Cbl carrier protein, human apo-transcobalamin II, reduced the required concentration of Cbl by 100-1000-fold. Any chemical modification of Cbl decreased its ability to support cellular viability and proliferation, with several of the modifications abrogating activity completely. All of the Cbl analogues that promoted growth required the presence of apo-transcobalamin II for the optimal support of cell growth. Generally, Cbl analogues modified at the *d*-position of the corrin ring and, to a lesser degree, analogues modified at the *b*- position supported cell growth, whereas analogues with modifications at the *e*-position did not support cell growth. Mixing experiments demonstrated an inverse order of potency of Cbl analogues to inhibit cell growth. Thus, Cbl analogues with modifications at the *e*-position were potent inhibitors, whereas *b*-analogues exhibited only partial inhibitory activity at high molar excess, and *d*-analogues had no inhibitory activity at all. These results indicate that modifications at the *e*-position of Cbl abolish the ability of Cbl to support cell growth and generate potent inhibitors of Cbl-dependent cell growth.

INTRODUCTION

CN-Cbl[2] is a water-soluble vitamin (vitamin B_{12}) that is essential for cell growth. Naturally occurring Cbl analogues are required as coenzymes by two mammalian enzymes that catalyze metabolically critical monocarbon transfer reactions (1). One reaction involves the methylation of homocysteine in the *de novo* synthesis of methionine and is catalyzed by methionine synthase. The other reaction rearranges L-methylmalonyl-CoA to succinyl-CoA and is catalyzed by L-methylmalonyl-CoA mutase. Cbl-binding proteins (R-binders and intrinsic factor) aid in its absorption from food and in its transport (2). The cellular uptake of Cbl is facilitated by the plasma protein TCII (3), which, when complexed to Cbl, binds to specific high affinity receptors on the surface of cells (4). The Cbl-TCII complex is internalized by receptor-mediated endocytosis, and Cbl is thought to be released from TCII via lysosomal action, followed by enzymatic modification to the forms that are active as coenzymes (1).

In humans, deficiencies of the vitamin or perturbations of its intracellular metabolism can result in a variety of cell growth-related disorders, including megaloblastic anemia, methylmalonic aciduria, and central nervous system abnormalities due to the improper functioning of the Cbl-dependent enzymes (5, 6). Cbl deficiency may be brought on by a lack of dietary Cbl, dysfunction of Cbl uptake via abnormalities in the binding proteins, including TCII (7), or errors of intracellular Cbl metabolism (8). Because Cbl deficiency can result in decreased cell proliferation, as evidenced in megaloblastic anemia, we have been investigating new methods to interfere with Cbl metabolism as part of a program to develop antiproliferative agents.

There are many naturally occurring analogues of Cbl (9), as well as a variety of Cbl analogues that have been synthesized by different laboratories (10, 11). Analysis of Cbl analogues *in vitro* and *in vivo* have shown that some interfere with Cbl metabolism, as evidenced by increased levels of homocysteine and methylmalonic acid, the substrates of the two mammalian enzymes dependent on Cbl (10, 12). More recently, it has been shown that relatively high doses of the c-lactam of CN-Cbl can inhibit the *in vitro* growth of HL60 cells (13), further promoting Cbl metabolism as a potential antiproliferative target.

In vitro cultures in which growth is dependent on Cbl have been reported (14, 15). Recently, we have described *in vitro* growth conditions in which the proliferation of human and murine leukemia cells were dependent on Cbl and recombinant human TCII (16). We have used this Cbl/TCII-dependent proliferation assay to evaluate the changes in growth characteristics of leukemic cells brought about by modifications in the chemical structure of Cbl. Here, we show that the modification of Cbl generally resulted in reduced ability to support cell growth. In particular, modifications of the propionamide side chains of the Cbl corrin ring resulted in reduced or complete loss of activity. In many cases, the loss of bioactivity of Cbl analogues correlated with a capacity to inhibit Cbl-dependent cell proliferation in a dose-dependent manner.

MATERIALS AND METHODS

Materials. BW5147 mouse lymphoma cells were obtained from American Type Culture Collection (Rockville, MD). RPMI 1640 culture medium and RPMI 1640 culture medium deficient in Cbl and folate were obtained from Stem Cell Technologies (Vancouver, Canada). FCS was from Life Technologies, Inc. (Grand Island, NY). QUSO was a gift from Degussa Corp. (Ridgefield, NJ). CN-Cbl, 5-methyl tetrahydrofolate, MTT, and DL-homocysteine were obtained from Sigma Chemical Co. (St. Louis, MO).

Recombinant Human TCII. Recombinant protein (apo form), kindly provided by E. V. Quadros (Veteran Affairs Medical Center and State University of New York Health Science Center, Brooklyn, NY), had been produced by infection of SF9 cells with baculovirus containing human TCII cDNA and purified as described (17).

Cell Culture. BW5147 cells were maintained in complete RPMI 1640 medium supplemented with 10% FCS. Cells were grown in 60 × 15 mm culture dishes (Fisher Scientific Co., Nepean, Canada) in a humidified atmosphere (5% CO_2, 95% air) at 37°C. Cells used in the Cbl/TCII bioassay were grown to late logarithmic phase, then washed three times in PBS before resuspension in Cbl-deficient bioassay medium.

Cbl/TCII Bioassay. To measure Cbl/TCII-dependent cell growth, we used RPMI 1640 deficient in Cbl and in which the folic acid was replaced with 1 μM 5-methyltetrahydrofolate and 1 μM homocysteine. FCS was pretreated with QUSO to reduce interference of endogenous bovine TCII/Cbl in the bioassay (16). In brief, 30 mg of QUSO were added per ml of FCS, mixed well, and removed by centrifugation as described previously (18). Washed cells were

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[1] This work was supported by Receptagen Corp. and the Medical Research Council of Canada.

[2] To whom requests for reprints should be addressed, at The Biomedical Research Centre, 2222 Health Sciences Mall, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3. Phone: (604) 822-7834; Fax: (604) 822-7815; E-mail: hermann@brc.ubc.ca.

[3] The abbreviations used are: CN-Cbl, cyanocobalamin; Cbl, cobalamin; TCII, transcobalamin II; QUSO, microfine precipitated silica; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Hepp, methylpurasil.

Source: Ex. 2058 at 4015; Paper 48 at 28.

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Sofyina v. Arsenyan

UDC 615.277.3:577.134.181

POSSIBLE AMPLIFICATION OF THE ANTINEOPLASTIC ACTION OF A FOLIC ACID ANTAGONIST BY METHYLCOBALAMINE ANALOGS

Z. P. SOFYINA, N. V. MYASISHCHEVA, F. G. ARSENYAN, A. M. YURKEVICH

Oncology Research Center of the USSR Academy of Medicine, Moscow

animal species (Rous sarcoma of chickens, PW-2 fibrosarcoma, sarcoma 45, and SSR [spontaneous sarcoma of rats] of rats, and Guerin's carcinoma, sarcoma 180, and lymphosarcoma of mice) and the attenuation of the curative effect of certain antineoplastic drugs in combined application with vitamin B_{12}, noted in early studies, are caused by the active biosynthesis of its coenzymes in the animals' bodies. Assessment of the functional role of methylcobalamine, one of the cobalamine coenzymes in the growth processes of normal and tumor cells, has drawn the greatest attention.

Methylcobalamine is a coenzyme of the methionine synthetase reaction, a key link defining the synergy of the action of cobalamines and folic acid compounds in cell proliferation processes. The special importance of methylcobalamine for activation of this enzyme system has been noted by a study of the disrupted metabolism of cobalamines in human leukoses. The poor effectiveness of combined cytostatic therapy in certain forms of acute leukosis involving high methylcobalamine concentrations in the blood has confirmed the specificity of its action in the body (Myasishcheva *et al.*, 1969). The active role of methylcobalamine in cell proliferation processes of hematopoietic tissue in healthy animals has now been established. Methylcobalamine increases the number of cells synthesizing DNA, their mitotic activity, and the size of the proliferative pool in the spleen of mice (Golenko *et al.*). A significant increase in the frequency of hemoblastosis development in mice has been found upon combined administration of methylcobalamine with endogenous blastomogens. An important point in the mechanism of the stimulant action of cobalamines is their inductive effect on methionine synthetase activity. In cultures of normal mammalian cells and human tumor cells, methionine synthetase activity rises noticeably with an increase in cobalamine concentration in the culture medium (Mangum *et al.*; Kamely *et al.*). However, various types of tumor cells differ from normal cells in their ability, on exposure to cobalamines, to increase the biosynthesis of methionine needed for rapid growth (Halpern *et al.*; Chello and Bertino). The salvage pathway with the aid of cobalamine-dependent methionine synthetase, which increases the intracellular pool of tetrahydrofolic acid independently of the folate reductase system, is evidently the principal mechanism of development of methotrexate (MTX) resistance in leukosis cells (Myasishcheva; Sauer and Jacnicke).

In this connection, there is a real possibility of amplifying the antineoplastic effect of this metabolite by combined application

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F. G. Arsenyan, N. V. Myasishcheva, Z. P. Sof'ina, M. O. Raushenbakh, I. P. Rudakova, E. G. Chauser, and A. M. Yurkevich

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 B_{12}, μg/kg	Increase in tumor volume after administration of methylcobalamin, % of control	
			1-6th day*	15-14th day*
Ca-755	BDF$_1$	10	−20	−10†
	C57BL	10	−26†	+10†
		500	+45	+51
		500	−37	+20†
AKATOL	BALB/c	10	+196	−37
RSHM-5	CBA	10	+87	0
Sarcoma 37	SHK	500	−67	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.

Oncological Scientific Center of the Academy of Medical Sciences of the USSR, Scientific-Industrial Vitamin Combine, Moscow. Translated from Khimiko-Farmatsevticheskii Zhurnal, Vol. 12, No. 10, pp. 49-54,

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Source: Ex. 2041 at 1, Ex. 2055 at 1299; Paper 48 at 28.

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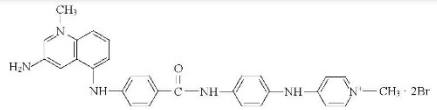
with cobalamine coenzyme antagonists. An under
action formed the basis for directed synthesis of m
potential antineoplastic compounds.

In chemotherapeutic experiments, we studied
cobalamine chloropalladate, which had exhibited
growth and inhibiting DNA synthesis in human en
al., 1977).

In developing a scheme of their combined act
physiologic action of cobalamines in the body: mo
cells and the formation of folate coenzymes, as wel
mor cells (Burke *et al.*; Tisman and Herbert; Flood
on the selective action of the studied compounds
pendent enzyme activity in the body. However, it w
their isolated application. Therefore, we thought it
of these compounds in the context of inhibition of dihydrofolate reductase activity using MTX.

Materials and Methods. The studies were conducted on mice of the C_{57}BL, CBA, BALB/c lines and BDF$_1$/C_{57}BL × DBA(2) hybrids weighing 20–25 g, obtained from the USSR Academy of Medicine nursery. The antineoplastic activity of methylcobalamine analogs was studied on transplanted leukoses L-1210 and La and on solid tu-



We obtained the drug from the U.S. National Cancer Institute in accordance with a U.S.–USSR agreement on cooperation in the area of tumor chemotherapy. According to the description provided by the American scientists, the drug is a methionine synthetase inhibitor (Carter *et al.*). The quinoline derivative was administered intraabdominally at 5 mg/kg daily or at 96-hour intervals, which corresponds to half the maximum tolerable dosage for the conditions. Treatment was begun 48 h after transplantation of the tumor. The results of the exposure were assessed 24 h after the end of the course of treatment and at various times throughout the animals' lives. Efficacy was measured by the percentage retardation of tumor growth, calculated by the conventional volume, and by the increase in the animals' lifespan. In each test, control and experimental groups were created so that their numbers would afford statistically significant minimum calculated percentage retardations

Lilly Ex. 2041 pg. 2
Neptune v. Lilly IPR2016-00237

The experiments were conducted on mice of the C_{57}BL, CBA, and BALB/c lines, the hybrids BDF$_1$-(C_{57}BL× DBA/2), F$_1$(C_{58}BL × 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 [16, 14].

Materials and Methods. The studies were conducted on mice of the C_{57}BL, CBA, BALB/c lines and BDF$_1$/C_{57}BL × DBA(2) hybrids weighing 20–25 g, obtained from the USSR Academy of Medicine nursery. The antineoplastic activity of methylcobalamine analogs was studied on transplanted leukoses L-1210 and La and on solid tumors: mammary adenocarcinoma (Ca-755), cervical uterine cancer (CUC-5), and intestinal adenocarcinoma (ACATOL). As the principal object of study, we selected solid tumors, on which it is easier to detect the stimulant effect

ll), 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 [15], 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

tumor. The antineoplastic
ous periods over the subsequent

th, calculated according to its
re subjected to statistical treat-

ally stimulates the growth of
l sarcoma 37 (Table 1).

tion, 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 μg/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 μg/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 (51–0%).

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,

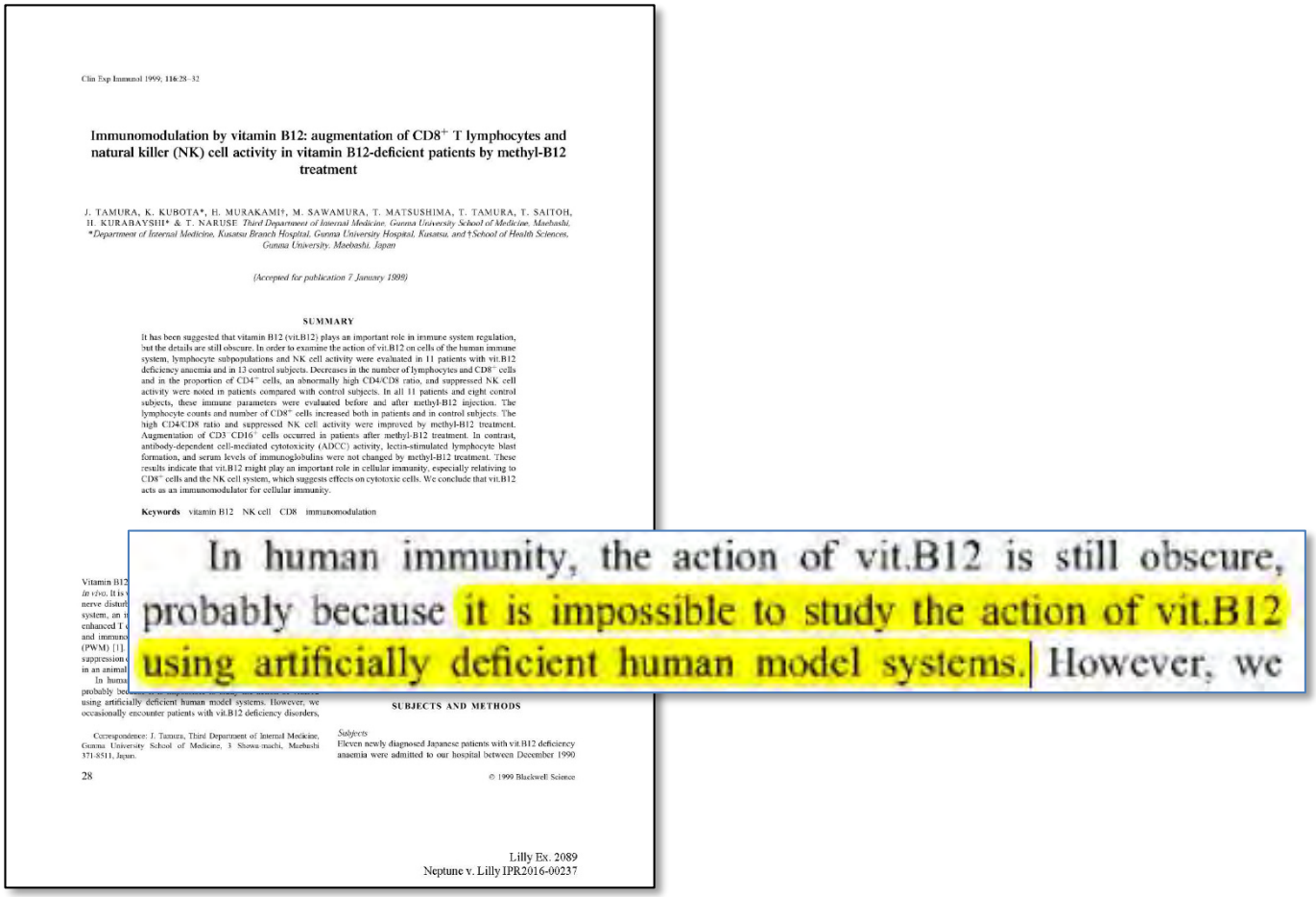
1301

Lilly Ex. 2055
Neptune v. Lilly IPR2016-00237

Source: Ex. 2041 at 2, Ex. 2055 at 1301; Paper 48 at 28.

State of the Art

Tamura



Clin Exp Immunol 1999; 116:28–32

Immunomodulation by vitamin B12: augmentation of CD8$^+$ T lymphocytes and natural killer (NK) cell activity in vitamin B12-deficient patients by methyl-B12 treatment

J. TAMURA, K. KUBOTA*, H. MURAKAMI†, M. SAWAMURA, T. MATSUSHIMA, T. TAMURA, T. SAITOH, H. KURABAYSHI* & T. NARUSE *Third Department of Internal Medicine, Gunma University School of Medicine, Maebashi, *Department of Internal Medicine, Kusatsu Branch Hospital, Gunma University Hospital, Kusatsu, and †School of Health Sciences, Gunma University, Maebashi, Japan*

(Accepted for publication 7 January 1998)

SUMMARY

It has been suggested that vitamin B12 (vit.B12) plays an important role in immune system regulation, but the details are still obscure. In order to examine the action of vit.B12 on cells of the human immune system, lymphocyte subpopulations and NK cell activity were evaluated in 11 patients with vit.B12 deficiency anaemia and in 13 control subjects. Decreases in the number of lymphocytes and CD8$^+$ cells and in the proportion of CD4$^+$ cells, an abnormally high CD4/CD8 ratio, and suppressed NK cell activity were noted in patients compared with control subjects. In all 11 patients and eight control subjects, these immune parameters were evaluated before and after methyl-B12 injection. The lymphocyte counts and number of CD8$^+$ cells increased both in patients and in control subjects. The high CD4/CD8 ratio and suppressed NK cell activity were improved by methyl-B12 treatment. Augmentation of CD3$^-$ CD16$^+$ cells occurred in patients after methyl-B12 treatment. In contrast, antibody-dependent cell-mediated cytotoxicity (ADCC) activity, lectin-stimulated lymphocyte blast formation, and serum levels of immunoglobulins were not changed by methyl-B12 treatment. These results indicate that vit.B12 might play an important role in cellular immunity, especially relativing to CD8$^+$ cells and the NK cell system, which suggests effects on cytotoxic cells. We conclude that vit.B12 acts as an immunomodulator for cellular immunity.

Keywords vitamin B12 NK cell CD8 immunomodulation

In human immunity, the action of vit.B12 is still obscure, probably because it is impossible to study the action of vit.B12 using artificially deficient human model systems. However, we

Vitamin B12 *In vivo*. It is nerve disturb system, an i enhanced T and immuno (PWM) [1]. suppression in an animal In huma probably be using artificially deficient human model systems. However, we occasionally encounter patients with vit.B12 deficiency disorders,

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SUBJECTS AND METHODS

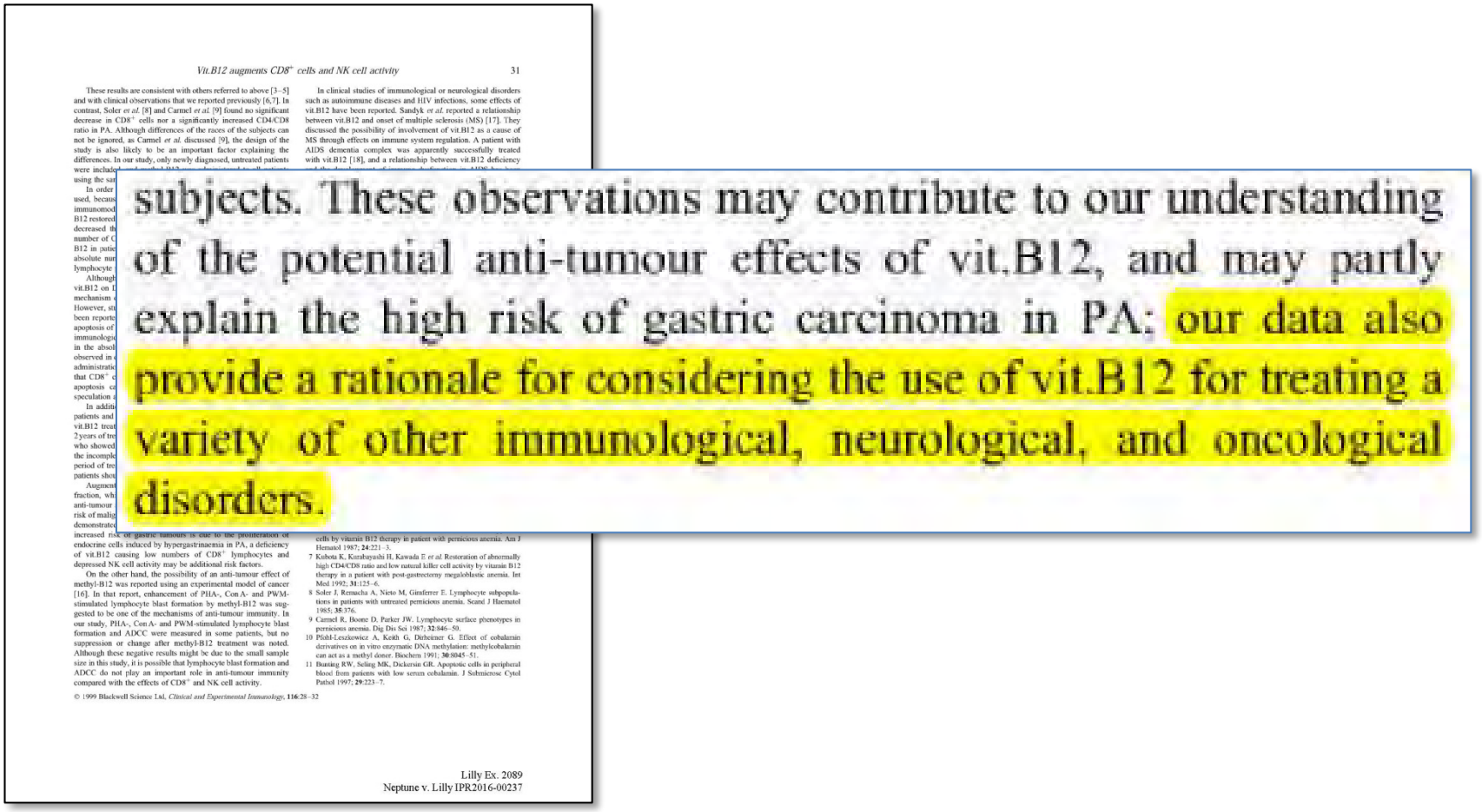
Subjects

Eleven newly diagnosed Japanese patients with vit.B12 deficiency anaemia were admitted to our hospital between December 1990

Source: Ex. 2089 at 28; Paper 48 at 28.

State of the Art

Tamura



Vit.B12 augments CD8⁺ cells and NK cell activity 31

These results are consistent with others referred to above [3–5] and with clinical observations that we reported previously [6,7]. In contrast, Soler *et al.* [8] and Carmel *et al.* [9] found no significant decrease in $CD8^+$ cells nor a significantly increased CD4/CD8 ratio in PA. Although differences of the races of the subjects can not be ignored, as Carmel *et al.* discussed [9], the design of the study is also likely to be an important factor explaining the differences. In our study, only newly diagnosed, untreated patients were included

In clinical studies of immunological or neurological disorders such as autoimmune diseases and HIV infections, some effects of vit.B12 have been reported. Sandyk *et al.* reported a relationship between vit.B12 and onset of multiple sclerosis (MS) [17]. They discussed the possibility of involvement of vit.B12 as a cause of MS through effects on immune system regulation. A patient with AIDS dementia complex was apparently successfully treated with vit.B12 [18], and a relationship between vit.B12 deficiency

subjects. These observations may contribute to our understanding of the potential anti-tumour effects of vit.B12, and may partly explain the high risk of gastric carcinoma in PA: our data also provide a rationale for considering the use of vit.B12 for treating a variety of other immunological, neurological, and oncological disorders.

endocrine cells induced by hypergastrinaemia in PA, a deficiency of vit.B12 causing low numbers of $CD8^+$ lymphocytes and depressed NK cell activity may be additional risk factors.

On the other hand, the possibility of an anti-tumour effect of methyl-B12 was reported using an experimental model of cancer [16]. In that report, enhancement of PHA-, Con A- and PWM-stimulated lymphocyte blast formation by methyl-B12 was suggested to be one of the mechanisms of anti-tumour immunity. In our study, PHA-, Con A- and PWM-stimulated lymphocyte blast formation and ADCC were measured in some patients, but no suppression or change after methyl-B12 treatment was noted. Although these negative results might be due to the small sample size in this study, it is possible that lymphocyte blast formation and ADCC do not play an important role in anti-tumour immunity compared with the effects of $CD8^+$ and NK cell activity.

 Clinical and Experimental Immunology, **116**:28–32

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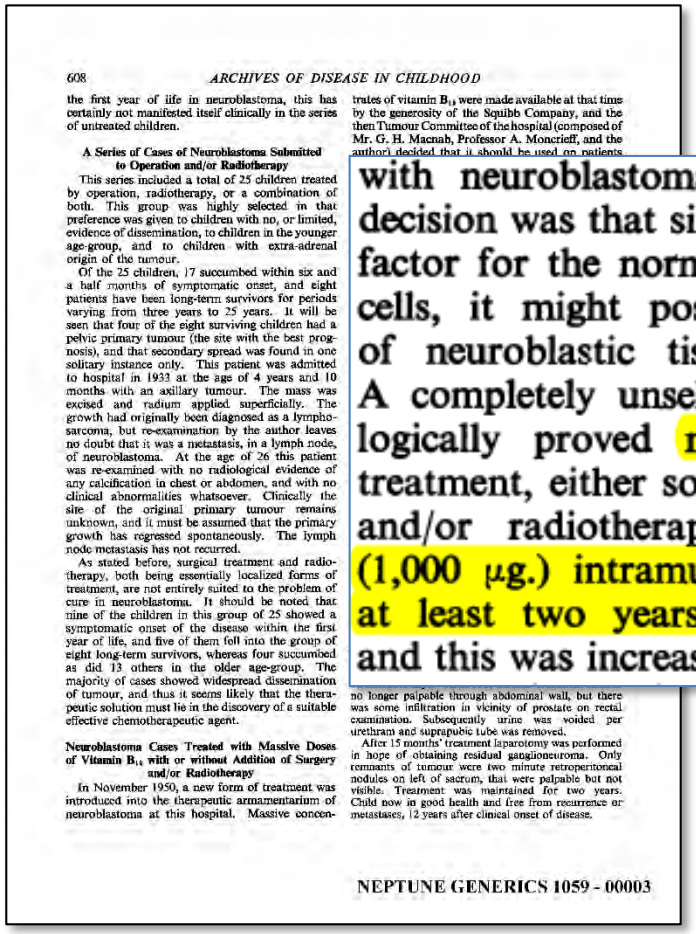
Lilly Ex. 2089
Neptune v. Lilly IPR2016-00237

Source: Ex. 2089 at 31; Paper 48 at 28.

State of the Art

Bodian 1963



608 ARCHIVES OF DISEASE IN CHILDHOOD

the first year of life in neuroblastoma, this has certainly not manifested itself clinically in the series of untreated children.

A Series of Cases of Neuroblastoma Submitted to Operation and/or Radiotherapy

This series included a total of 25 children treated by operation, radiotherapy, or a combination of both. This group was highly selected in that preference was given to children with no, or limited, evidence of dissemination, to children in the younger age-group, and to children with extra-adrenal origin of the tumour.

Of the 25 children, 17 succumbed within six and a half months of symptomatic onset, and eight patients have been long-term survivors for periods varying from three years to 25 years. It will be seen that four of the eight surviving children had a pelvic primary tumour (the site with the best prognosis), and that secondary spread was found in one solitary instance only. This patient was admitted to hospital in 1933 at the age of 4 years and 10 months with an axillary tumour. The mass was excised and radium applied superficially. The growth had originally been diagnosed as a lymphosarcoma, but re-examination by the author leaves no doubt that it was a metastasis, in a lymph node, of neuroblastoma. At the age of 26 this patient was re-examined with no radiological evidence of any calcification in chest or abdomen, and with no clinical abnormalities whatsoever. Clinically the site of the original primary tumour remains unknown, and it must be assumed that the primary growth has regressed spontaneously. The lymph node metastasis has not recurred.

As stated before, surgical treatment and radiotherapy, both being essentially localized forms of treatment, are not entirely suited to the problem of cure in neuroblastoma. It should be noted that nine of the children in this group of 25 showed a symptomatic onset of the disease within the first year of life, and five of them fell into the group of eight long-term survivors, whereas four succumbed as did 13 others in the older age-group. The majority of cases showed widespread dissemination of tumour, and thus it seems likely that the therapeutic solution must lie in the discovery of a suitable effective chemotherapeutic agent.

Neuroblastoma Cases Treated with Massive Doses of Vitamin B_{12} with or without Addition of Surgery and/or Radiotherapy

In November 1950, a new form of treatment was introduced into the therapeutic armamentarium of neuroblastoma at this hospital. Massive concentrates of vitamin B_{12} were made available at that time by the generosity of the Squibb Company, and the then Tumour Committee of the hospital (composed of Mr. G. H. Macnab, Professor A. Moncrieff, and the author) decided that it should be used on patients with neuroblastoma. The reasoning behind this decision was that since vitamin B_{12} was an essential factor for the normal maturation of haemopoietic cells, it might possibly enhance the maturation of neuroblastic tissue towards ganglioneuroma. A completely unselected series of cases of histologically proved neuroblastoma was given this treatment, either solely or in addition to operation and/or radiotherapy. The dosage was 1 mg. (1,000 μg.) intramuscularly on alternate days for at least two years whenever survival permitted, and this was increased from 1957 onwards to 1 mg.

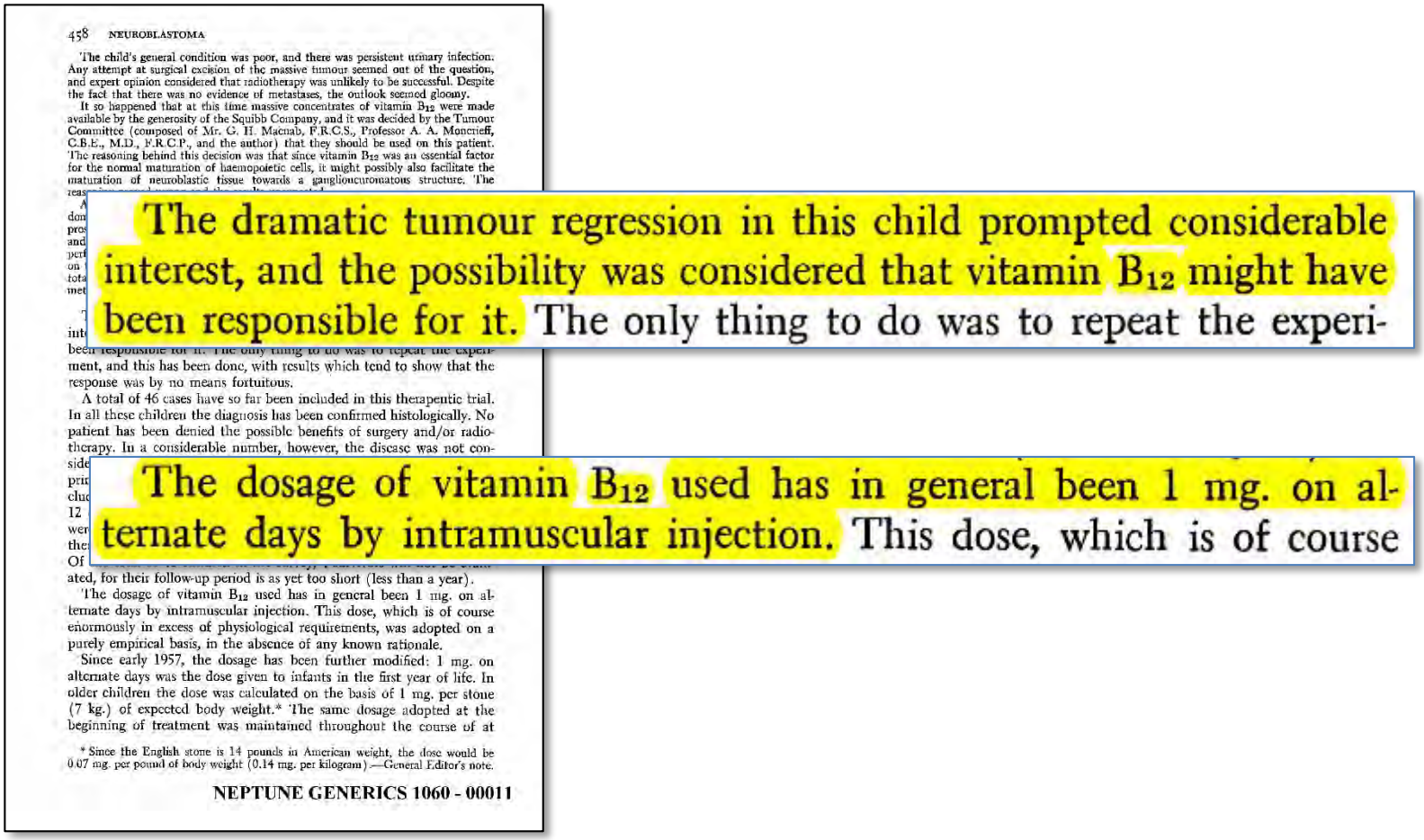
no longer palpable through abdominal wall, but there was some infiltration in vicinity of prostate on rectal examination. Subsequently urine was voided per urethram and suprapubic tube was removed.

After 15 months' treatment laparotomy was performed in hope of obtaining residual ganglioneuroma. Only remnants of tumour were two minute retroperitoneal nodules on left of sacrum, that were palpable but not visible. Treatment was maintained for two years. Child now in good health and free from recurrence or metastases, 12 years after clinical onset of disease.

NEPTUNE GENERICS 1059 - 00003

State of the Art

Bodian 1959



458 NEUROBLASTOMA

The child's general condition was poor, and there was persistent urinary infection. Any attempt at surgical excision of the massive tumour seemed out of the question, and expert opinion considered that radiotherapy was unlikely to be successful. Despite the fact that there was no evidence of metastases, the outlook seemed gloomy.

It so happened that at this time massive concentrates of vitamin B_{12} were made available by the generosity of the Squibb Company, and it was decided by the Tumour Committee (composed of Mr. G. H. Macnab, F.R.C.S., Professor A. A. Moncrieff, C.B.E., M.D., F.R.C.P., and the author) that they should be used on this patient. The reasoning behind this decision was that since vitamin B_{12} was an essential factor for the normal maturation of haemopoietic cells, it might possibly also facilitate the maturation of neuroblastic tissue towards a ganglioneuromatous structure. The

The dramatic tumour regression in this child prompted considerable interest, and the possibility was considered that vitamin B_{12} might have been responsible for it. The only thing to do was to repeat the experi-

ment, and this has been done, with results which tend to show that the response was by no means fortuitous.

A total of 46 cases have so far been included in this therapeutic trial. In all these children the diagnosis has been confirmed histologically. No patient has been denied the possible benefits of surgery and/or radiotherapy. In a considerable number, however, the disease was not con-

The dosage of vitamin B_{12} used has in general been 1 mg. on alternate days by intramuscular injection. This dose, which is of course

ated, for their follow-up period is as yet too short (less than a year).

The dosage of vitamin B_{12} used has in general been 1 mg. on alternate days by intramuscular injection. This dose, which is of course enormously in excess of physiological requirements, was adopted on a purely empirical basis, in the absence of any known rationale.

Since early 1957, the dosage has been further modified: 1 mg. on alternate days was the dose given to infants in the first year of life. In older children the dose was calculated on the basis of 1 mg. per stone (7 kg.) of expected body weight.* The same dosage adopted at the beginning of treatment was maintained throughout the course of at

* Since the English stone is 14 pounds in American weight, the dose would be 0.07 mg. per pound of body weight (0.14 mg. per kilogram).—General Editor's note.

NEPTUNE GENERICS 1060 - 00011

State of the Art

Lockwood

with gamma-linolenic acid. The clinical application of anti-oxidants has been reviewed by Noto *et al.* (1989) and by Floyd (1990).

Kneckt *et al.* (1990) found in an epidemiological study an 11 times higher risk of breast cancer with low selenium and vitamin E in conjunction. Preliminary results from intervention studies seem to yield similar findings (Blot *et al.*, 1993; Adjuvant Nutrition in Cancer Treatment (Symposium, 1992).

These studies on vitamins and nutritional entities and their relationship to the prevention and treatment of cancer were some of more than 200 references forming the basis for our protocol on the treatment of breast cancer with nutritional entities, and particularly vitamin Q_{10}.

Thirty-two women with breast cancer in the so-called high risk group were included in an open and still ongoing trial, for which they gave their informed consent.

This report is based on an 18 months follow-up study. For ethical reasons, and anticipating lack of compliance with the large number of supplements, the trial was open and aimed towards a finding of a possible positive response, which would then be a basis for a blinded trial.

All patients were treated according to the routine procedure in Denmark, i.e. surgery, chemotherapy, X-ray treatment and in some cases Tamoxifen in accordance with the estrogen receptor status of the tumor.

The patients were between 32 and 81 years. Beside the spreading of cancer to the lymph nodes, some of the patients had metastases at different sites such as the skin, the pleura or in the thoracic vertebrae.

All patients underwent clinical check-ups every 3 months in order to detect any recurrence of the disease. Mammography, bone scan and X-ray pictures of the chest or spine were performed whenever there was any suspicion of recurrence. Open biopsies or 'Trucut (R)' biopsies were also performed. Blood pressure, body weight, use of painkillers and quality of life parameters were followed.

At 0, 3 and 12 months, blood tests of Coenzyme Q_{10} (whole blood) were obtained, in order to follow compliance. For a random subgroup of 1/3, extensive hematological, immunological and nutritional parameters were followed including whole blood Q_{10},

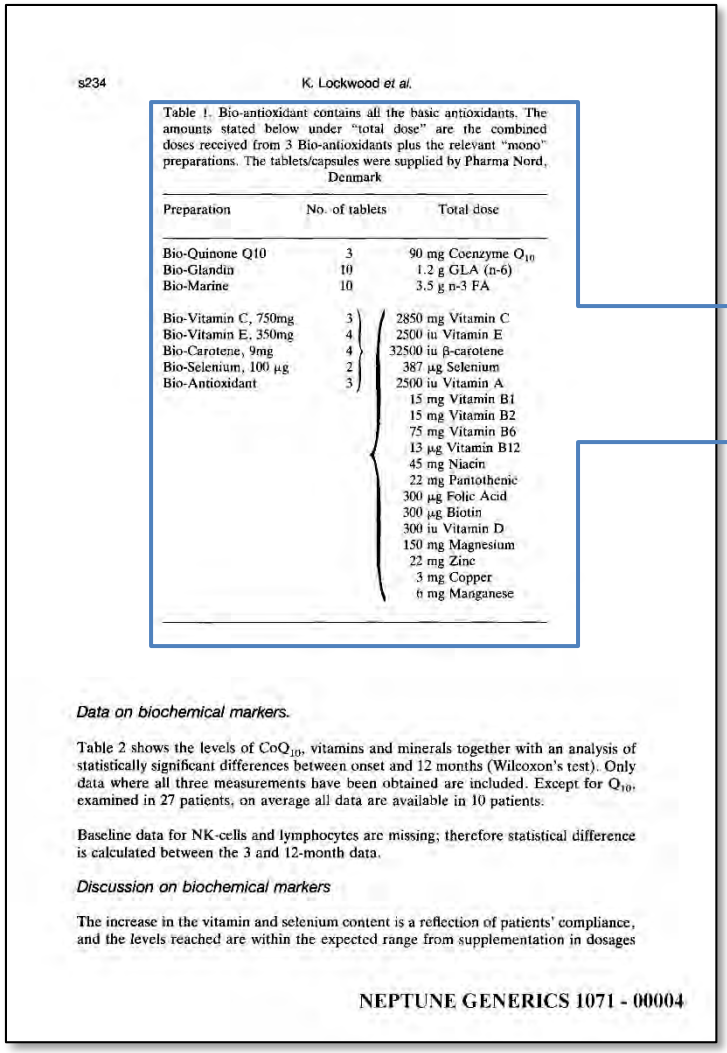
All patients took the following supplements in a daily pack divided morning and evening (Table 1.)

NEPTUNE GENERICS 1071 - 00003

Source: Ex. 1071 at S233; Paper 48 at 28.

State of the Art

Lockwood



s234 K. Lockwood et al.

Table 1. Bio-antioxidant contains all the basic antioxidants. The amounts stated below under "total dose" are the combined doses received from 3 Bio-antioxidants plus the relevant "mono" preparations. The tablets/capsules were supplied by Pharma Nord, Denmark

Preparation	No. of tablets	Total dose
Bio-Quinone Q10	3	90 mg Coenzyme Q_{10}
Bio-Glandin	10	1.2 g GLA (n-6)
Bio-Marine	10	3.5 g n-3 FA
Bio-Vitamin C, 750mg	3	2850 mg Vitamin C
Bio-Vitamin E, 350mg	4	2500 iu Vitamin E
Bio-Carotene, 9mg	4	32500 iu β-carotene
Bio-Selenium, 100 µg	2	387 µg Selenium
Bio-Antioxidant	3	2500 iu Vitamin A
		15 mg Vitamin B1
		15 mg Vitamin B2
		75 mg Vitamin B6
		13 µg Vitamin B12
		45 mg Niacin
		22 mg Pantothenic
		300 µg Folic Acid
		300 µg Biotin
		300 iu Vitamin D
		150 mg Magnesium
		22 mg Zinc
		3 mg Copper
		6 mg Manganese

Data on biochemical markers.

Table 2 shows the levels of CoQ_{10}, vitamins and minerals together with an analysis of statistically significant differences between onset and 12 months (Wilcoxon's test). Only data where all three measurements have been obtained are included. Except for Q_{10}, examined in 27 patients, on average all data are available in 10 patients.

Baseline data for NK-cells and lymphocytes are missing; therefore statistical difference is calculated between the 3 and 12-month data.

Discussion on biochemical markers

The increase in the vitamin and selenium content is a reflection of patients' compliance, and the levels reached are within the expected range from supplementation in dosages

NEPTUNE GENERICS 1071 - 00004

Table 1. Bio-antioxidant contains all the basic antioxidants. The amounts stated below under "total dose" are the combined doses received from 3 Bio-antioxidants plus the relevant "mono" preparations. The tablets/capsules were supplied by Pharma Nord, Denmark

Preparation	No. of tablets	Total dose
Bio-Quinone Q10	3	90 mg Coenzyme Q_{10}
Bio-Glandin	10	1.2 g GLA (n-6)
Bio-Marine	10	3.5 g n-3 FA
Bio-Vitamin C, 750mg	3	2850 mg Vitamin C
Bio-Vitamin E, 350mg	4	2500 iu Vitamin E
Bio-Carotene, 9mg	4	32500 iu β-carotene
Bio-Selenium, 100 µg	2	387 µg Selenium
Bio-Antioxidant	3	2500 iu Vitamin A
		15 mg Vitamin B1
		15 mg Vitamin B2
		75 mg Vitamin B6
		13 µg Vitamin B12
		45 mg Niacin
		22 mg Pantothenic
		300 µg Folic Acid
		300 µg Biotin
		300 iu Vitamin D
		150 mg Magnesium
		22 mg Zinc
		3 mg Copper
		6 mg Manganese

State of the Art

Barber

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Article no. bjoc.1999.0654

The effect of an oral nutritional supplement enriched with fish oil on weight-loss in patients with pancreatic cancer

MD Barber[1], JA Ross[1], AC Voss[2], MJ Tisdale[3] and KCH Fearon[1]

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Summary Previous studies have suggested that administration of oral eicosapentaenoic acid (EPA) will stabilize weight in patients with

supplement could produce weight gain in these patients. Twenty patients with unresectable pancreatic adenocarcinoma were asked to consume two cans of a fish oil-enriched nutritional supplement per day in addition to their normal food intake. Each can contained 310 kcal,

and 7 weeks (median 2 kg, $P = 0.033$). Dietary intake increased significantly by almost 400 kcal day^{-1} ($P = 0.002$). REE per kg body weight and per kg lean body mass fell significantly. Performance status and appetite were significantly improved at 3 weeks. In contrast to previous studies of oral conventional nutritional supplements in weight-losing cancer patients, this study suggests that an EPA-enriched supplement may reverse cachexia in advanced pancreatic cancer.

Keywords: pancreatic cancer; cachexia; eicosapentaenoic acid; docosahexaenoic acid; fish oil; nutritional supplementation

Pancreatic cancer is almost inevitably associated with progressive nutritional decline (Wigmore et al, 1997a). Weight loss in patients with gastrointestinal cancer is often refractory to therapeutic intervention and is associated with a shorter survival time and a reduced quality of life (DeWys et al, 1980; Ovesen et al, 1993a). The provision of conventional oral nutritional supplements may increase overall dietary intake but this does not generally lead to any benefit in terms of nutritional status (Evans et al, 1987; Ovesen et al, 1993b). Consequently it has been suggested that the metabolic processes which contribute to weight-loss in patients with cancer may also block the accretion of lean tissue (Moldawer and Copeland, 1997).

Pro-inflammatory cytokines, notably interleukin 6 (IL-6), can induce a cachectic state when injected into animals and monoclonal antibodies to such cytokines may attenuate certain features of cachexia in tumour-bearing animals (McNamara et al, 1992). The acute phase protein response (APPR) has been shown to be associated with increased resting energy expenditure (Falconer et al, 1994a) and to correlate with reduced nutritional intake (Wigmore et al, 1997b) in weight-losing patients with pancreatic cancer. In addition, the APPR has been demonstrated to be the strongest independent predictor of poor prognosis in patients with pancreatic cancer (Falconer et al, 1995). The APPR is modulated primarily by pro-inflammatory cytokines, including IL-6 (Heinrich et al, 1990). Thus there appears to be a strong link between pro inflammatory cytokine activity and the development of cachexia in patients with pancreatic cancer. Attempts to manipulate the inflammatory response in cancer patients with the intention of improving nutritional status have been made previously with promising results (McMillan et al, 1997).

The n-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are immunomodulatory and have been shown to suppress endotoxin-induced production of pro-inflammatory cytokines such as IL-1 and tumour necrosis factor (TNF) by peripheral blood mononuclear cells (PBMC) from healthy volunteers (Meydani et al, 1993). Studies of weight-losing pancreatic cancer patients receiving high-purity EPA have demonstrated suppression of PBMC IL-6 production (Wigmore et al, 1997c). EPA has also been shown to have inhibitory effects on the growth of human pancreatic cancer cell lines in vitro (Falconer et al, 1994b) and to have anti-tumour and anti-cachectic effects in the chemoresistant murine MAC16 colon adenocarcinoma model (Beck et al, 1991). The cachexia seen in this animal model has been attributed to the production of a proteolysis inducing factor by tumour cells and such a factor is also found in tumour-bearing humans with cachexia (Todorov et al, 1996). It has been suggested that EPA may act by inhibiting the end organ effects of this factor (Tisdale, 1996).

We have previously reported that administration of a mixed fish oil preparation (providing around 2.2 g EPA and 1.4 g DHA daily) and a pure EPA preparation (providing 6 g EPA daily) will stabilize weight in patients with unresectable pancreatic cancer (Wigmore et al, 1996; Barber et al, 1997). Clearly, in order to lay down new tissue and thereby increase body weight additional macronutrients need to be consumed.

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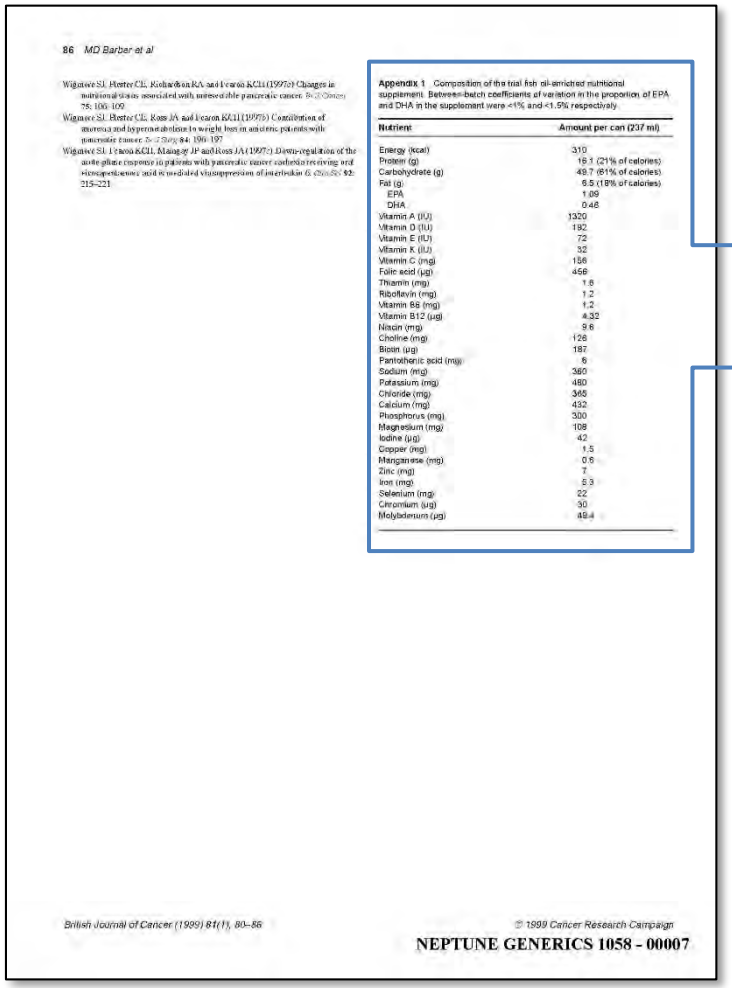
Correspondence to: KCH Fearon

Source: Ex. 1058 at 80; Paper 48 at 28.

State of the Art

Barber



86 MD Barber et al

Wigmore SJ, Plester CE, Richardson RA and Fearon KCH (1997a) Changes in nutritional status associated with unresectable pancreatic cancer. *Br J Cancer* 75: 100–109

Wigmore SJ, Plester CE, Ross JA and Fearon KCH (1997b) Contribution of anorexia and hypermetabolism to weight loss in anorexic patients with pancreatic cancer. *Nutr Cancer* 84: 196–197

Wigmore SJ, Fearon KCH, Maingay JP and Ross JA (1997c) Down-regulation of the acute phase response in patients with pancreatic cancer cachexia receiving oral eicosapentaenoic acid is mediated via suppression of interleukin-6. *Clin Sci* 92: 215–221

Appendix 1 Composition of the trial fish oil-enriched nutritional supplement. Between-batch coefficients of variation in the proportion of EPA and DHA in the supplement were <1% and <1.5% respectively

Nutrient	Amount per can (237 ml)
Energy (kcal)	310
Protein (g)	16.1 (21% of calories)
Carbohydrate (g)	49.7 (61% of calories)
Fat (g)	6.5 (18% of calories)
EPA	1.09
DHA	0.46
Vitamin A (IU)	1320
Vitamin D (IU)	192
Vitamin E (IU)	72
Vitamin K (IU)	32
Vitamin C (mg)	156
Folic acid (μg)	456
Thiamin (mg)	1.6
Riboflavin (mg)	1.2
Vitamin B6 (mg)	1.2
Vitamin B12 (μg)	4.32
Niacin (mg)	9.6
Choline (mg)	126
Biotin (μg)	187
Pantothenic acid (mg)	6
Sodium (mg)	360
Potassium (mg)	480
Chloride (mg)	365
Calcium (mg)	432
Phosphorus (mg)	300
Magnesium (mg)	108
Iodine (μg)	42
Copper (mg)	1.5
Manganese (mg)	0.6
Zinc (mg)	7
Iron (mg)	5.3
Selenium (mg)	22
Chromium (μg)	30
Molybdenum (μg)	49.4

British Journal of Cancer (1999) 81(1), 80–86

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Appendix 1 Composition of the trial fish oil-enriched nutritional supplement. Between-batch coefficients of variation in the proportion of EPA and DHA in the supplement were <1% and <1.5% respectively

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Iron (mg)	5.3
Selenium (mg)	22
Chromium (μg)	30
Molybdenum (μg)	49.4

Source: Ex. 1058 at 86; Paper 48 at 28.

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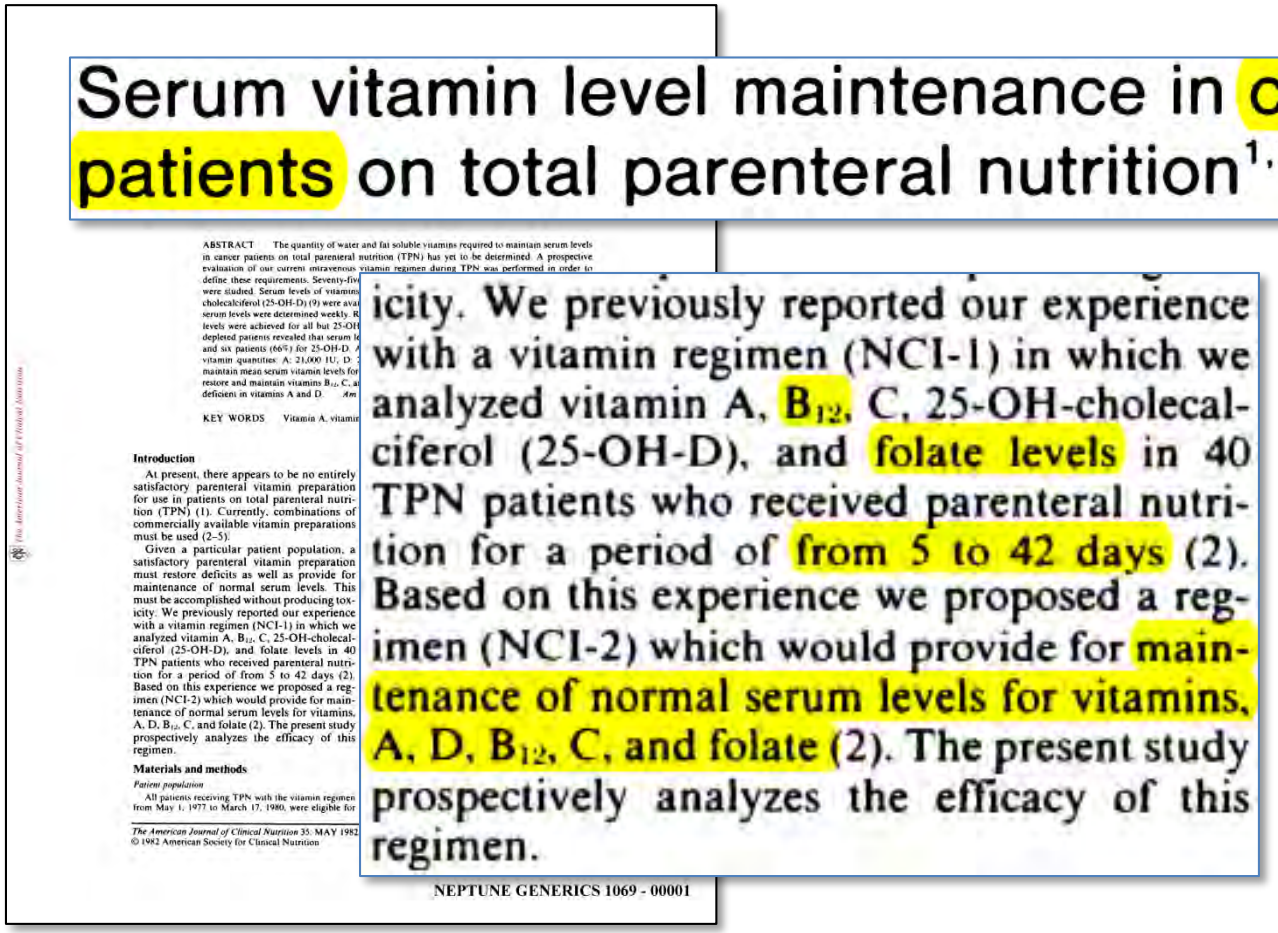

Serum vitamin level maintenance in cancer patients on total parenteral nutrition[1,2]

ABSTRACT The quantity of water and fat soluble vitamins required to maintain serum levels in cancer patients on total parenteral nutrition (TPN) has yet to be determined. A prospective evaluation of our current intravenous vitamin regimen during TPN was performed in order to define these requirements. Seventy-five were studied. Serum levels of vitamins cholecalciferol (25-OH-D) (9) were ava serum levels were determined weekly. R levels were achieved for all but 25-OH depleted patients revealed that serum le and six patients (66%) for 25-OH-D. A vitamin quantities: A: 21,000 IU, D: maintain mean serum vitamin levels for restore and maintain vitamins B_{12}, C, a deficient in vitamins A and D. *Am*

KEY WORDS Vitamin A, vitamin

Introduction

At present, there appears to be no entirely satisfactory parenteral vitamin preparation for use in patients on total parenteral nutrition (TPN) (1). Currently, combinations of commercially available vitamin preparations must be used (2–5).

Given a particular patient population, a satisfactory parenteral vitamin preparation must restore deficits as well as provide for maintenance of normal serum levels. This must be accomplished without producing toxicity. We previously reported our experience with a vitamin regimen (NCI-1) in which we analyzed vitamin A, B_{12}, C, 25-OH-cholecalciferol (25-OH-D), and folate levels in 40 TPN patients who received parenteral nutrition for a period of from 5 to 42 days (2). Based on this experience we proposed a regimen (NCI-2) which would provide for maintenance of normal serum levels for vitamins, A, D, B_{12}, C, and folate (2). The present study prospectively analyzes the efficacy of this regimen.

Materials and methods

Patient population

All patients receiving TPN with the vitamin regimen from May 1, 1977 to March 17, 1980, were eligible for

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icity. We previously reported our experience with a vitamin regimen (NCI-1) in which we analyzed vitamin A, B_{12}, C, 25-OH-cholecalciferol (25-OH-D), and folate levels in 40 TPN patients who received parenteral nutrition for a period of from 5 to 42 days (2). Based on this experience we proposed a regimen (NCI-2) which would provide for maintenance of normal serum levels for vitamins, A, D, B_{12}, C, and folate (2). The present study prospectively analyzes the efficacy of this regimen.

Source: Ex. 1069 at 1003; Paper 48 at 28.

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Niyikiza C, Baker S, Johnson R, Walling J, Seitz D, Allen R. 1998. MTA (LY231514): Relationship of vitamin metabolite profile, drug exposure, and other patient characteristics to toxicity. Annals of Oncology Supplement 4 to Vol 9, Abstract 609.

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Worzalla JF, Shih C, Schultz RM Role of folic acid in modulating the toxicity and efficacy of the multitargeted antifolate, LY231414 Anticancer Res 18 (5A):3235-3239 1998

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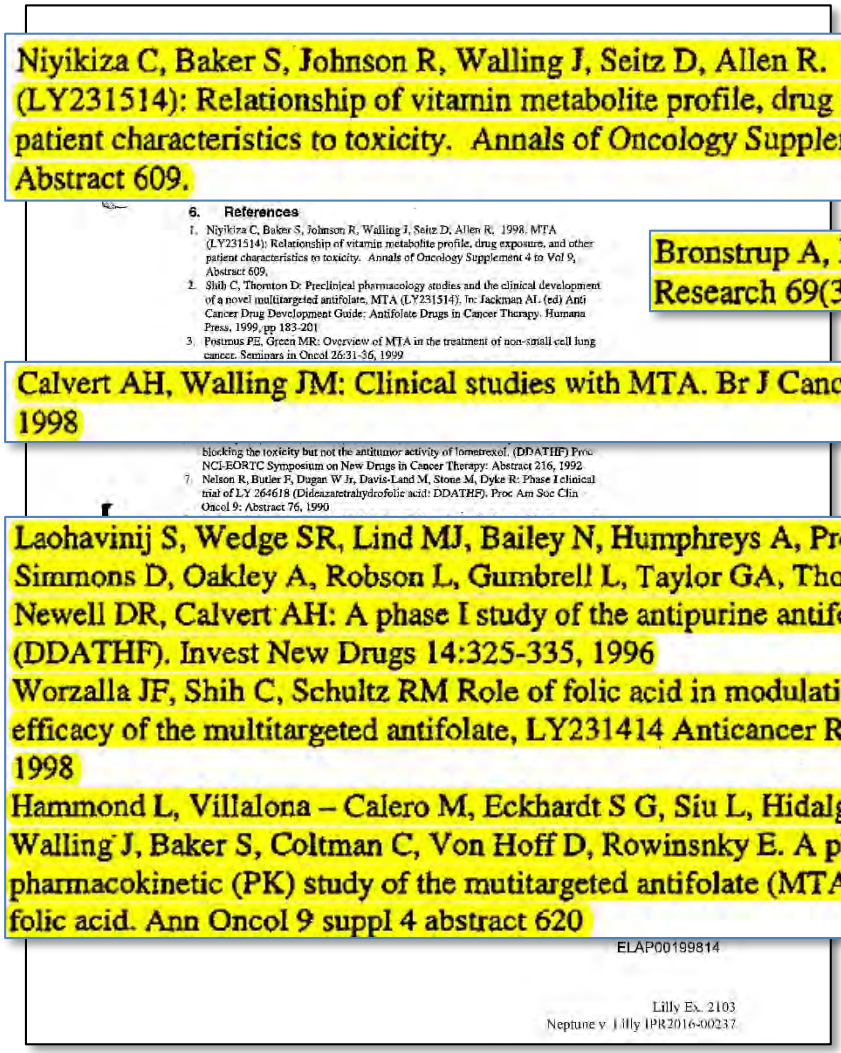
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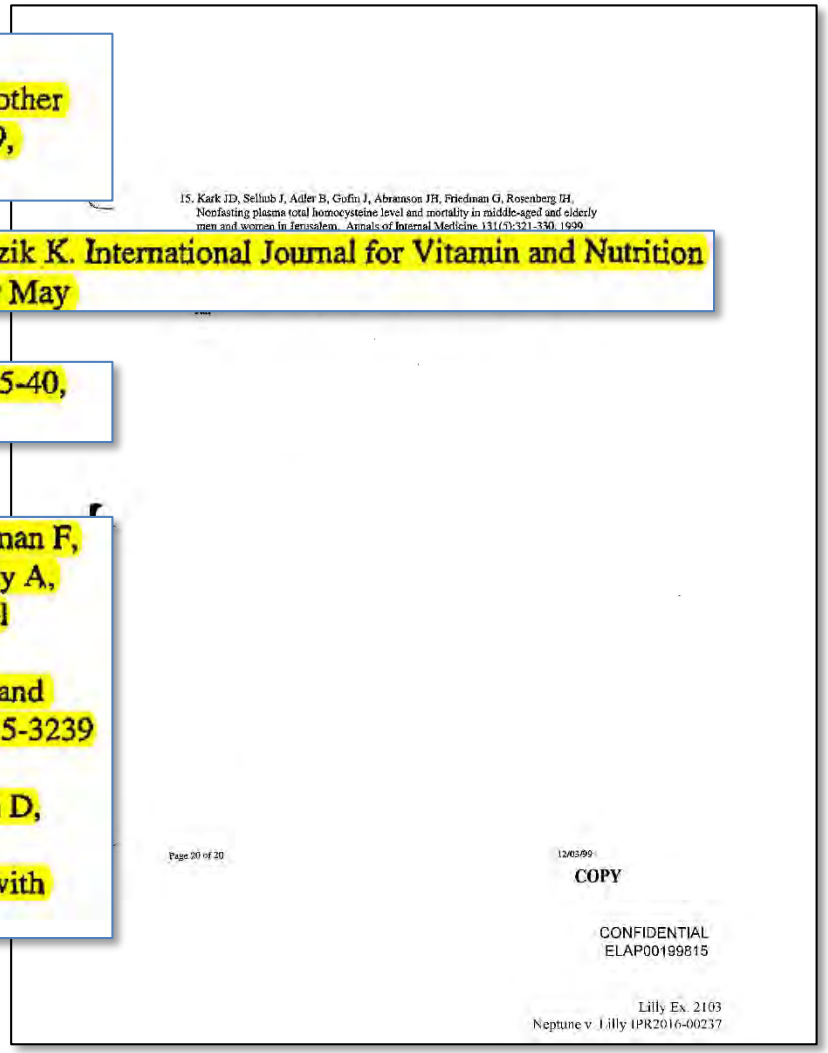
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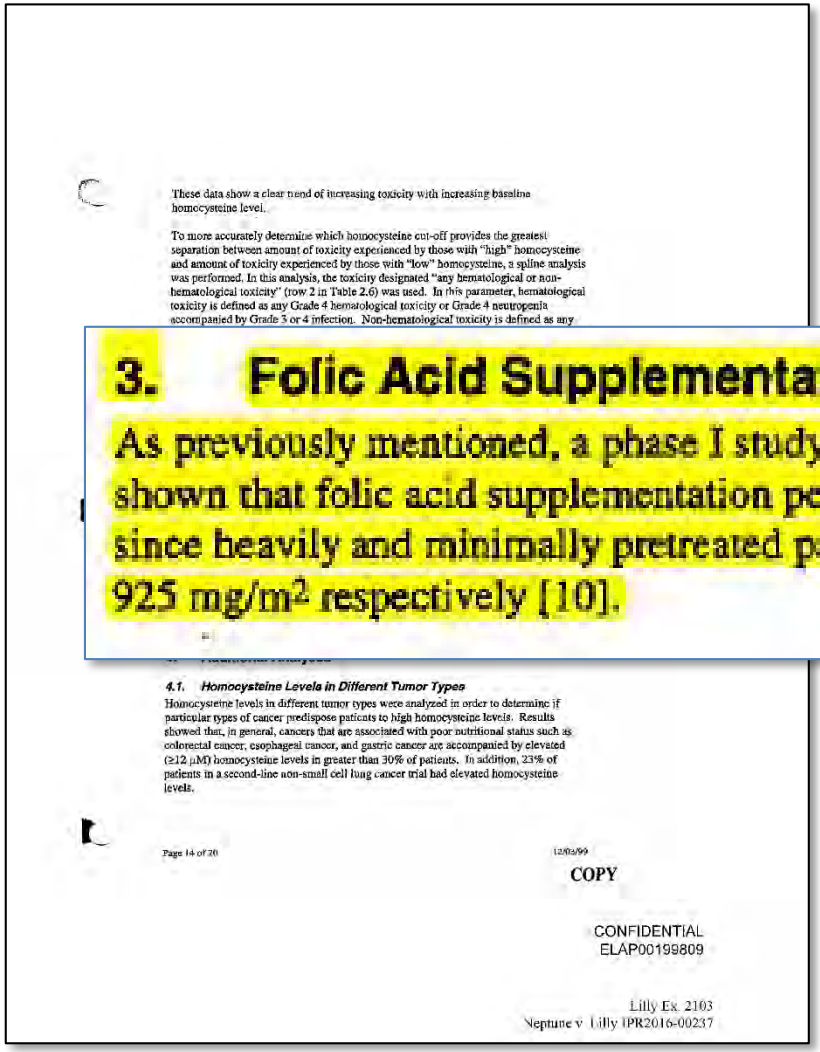

These data show a clear trend of increasing toxicity with increasing baseline homocysteine level.

To more accurately determine which homocysteine cut-off provides the greatest separation between amount of toxicity experienced by those with "high" homocysteine and amount of toxicity experienced by those with "low" homocysteine, a spline analysis was performed. In this analysis, the toxicity designated "any hematological or non-hematological toxicity" (row 2 in Table 2.6) was used. In this parameter, hematological toxicity is defined as any Grade 4 hematological toxicity or Grade 4 neutropenia accompanied by Grade 3 or 4 infection. Non-hematological toxicity is defined as any

3. Folic Acid Supplementation

As previously mentioned, a phase I study of LY231514 and folic acid (Study JMAS) has shown that folic acid supplementation permits dose escalation by ameliorating toxicity since heavily and minimally pretreated patients tolerate LY231514 at doses of 700 and 925 mg/m^2 respectively [10].

4.1. Homocysteine Levels in Different Tumor Types

Homocysteine levels in different tumor types were analyzed in order to determine if particular types of cancer predispose patients to high homocysteine levels. Results showed that, in general, cancers that are associated with poor nutritional status such as colorectal cancer, esophageal cancer, and gastric cancer are accompanied by elevated (≥12 μM) homocysteine levels in greater than 30% of patients. In addition, 23% of patients in a second-line non-small cell lung cancer trial had elevated homocysteine levels.

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Source: Ex. 2103 at 14; Paper 48 at 6, 17.

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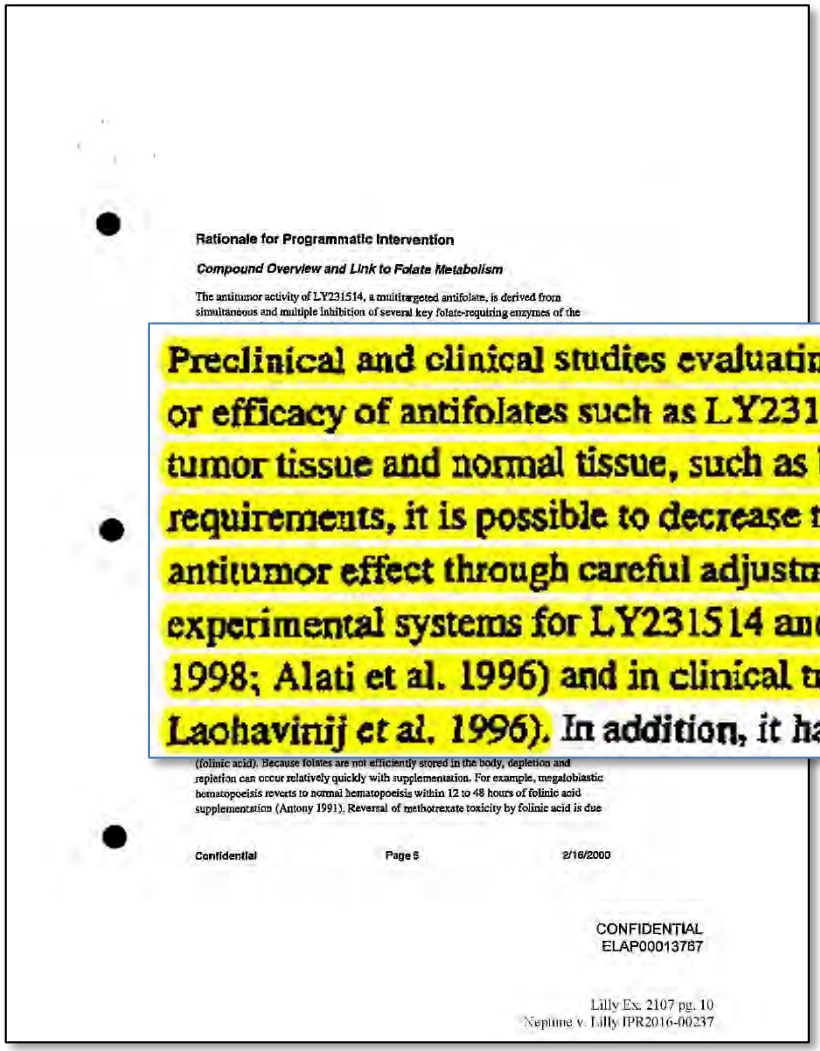

Rationale for Programmatic Intervention

Compound Overview and Link to Folate Metabolism

The antitumor activity of LY231514, a multitargeted antifolate, is derived from simultaneous and multiple inhibition of several key folate-requiring enzymes of the

(folinic acid). Because folates are not efficiently stored in the body, depletion and repletion can occur relatively quickly with supplementation. For example, megaloblastic hematopoeisis reverts to normal hematopoeisis within 12 to 48 hours of folinic acid supplementation (Antony 1991). Reversal of methotrexate toxicity by folinic acid is due

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Preclinical and clinical studies evaluating the impact of dietary folic acid on the toxicity or efficacy of antifolates such as LY231514 and lometrexol have been reported. Because tumor tissue and normal tissue, such as bone marrow, presumably have different folate requirements, it is possible to decrease the toxicity to healthy tissue while maintaining antitumor effect through careful adjustment of folic acid intake. This has been shown in experimental systems for LY231514 and another antifolate, lometrexol (Worzalla et al. 1998; Alati et al. 1996) and in clinical trials with lometrexol (Young et al. 1992; Laohavinij et al. 1996). In addition, it has been clinically observed that the efficacy of low

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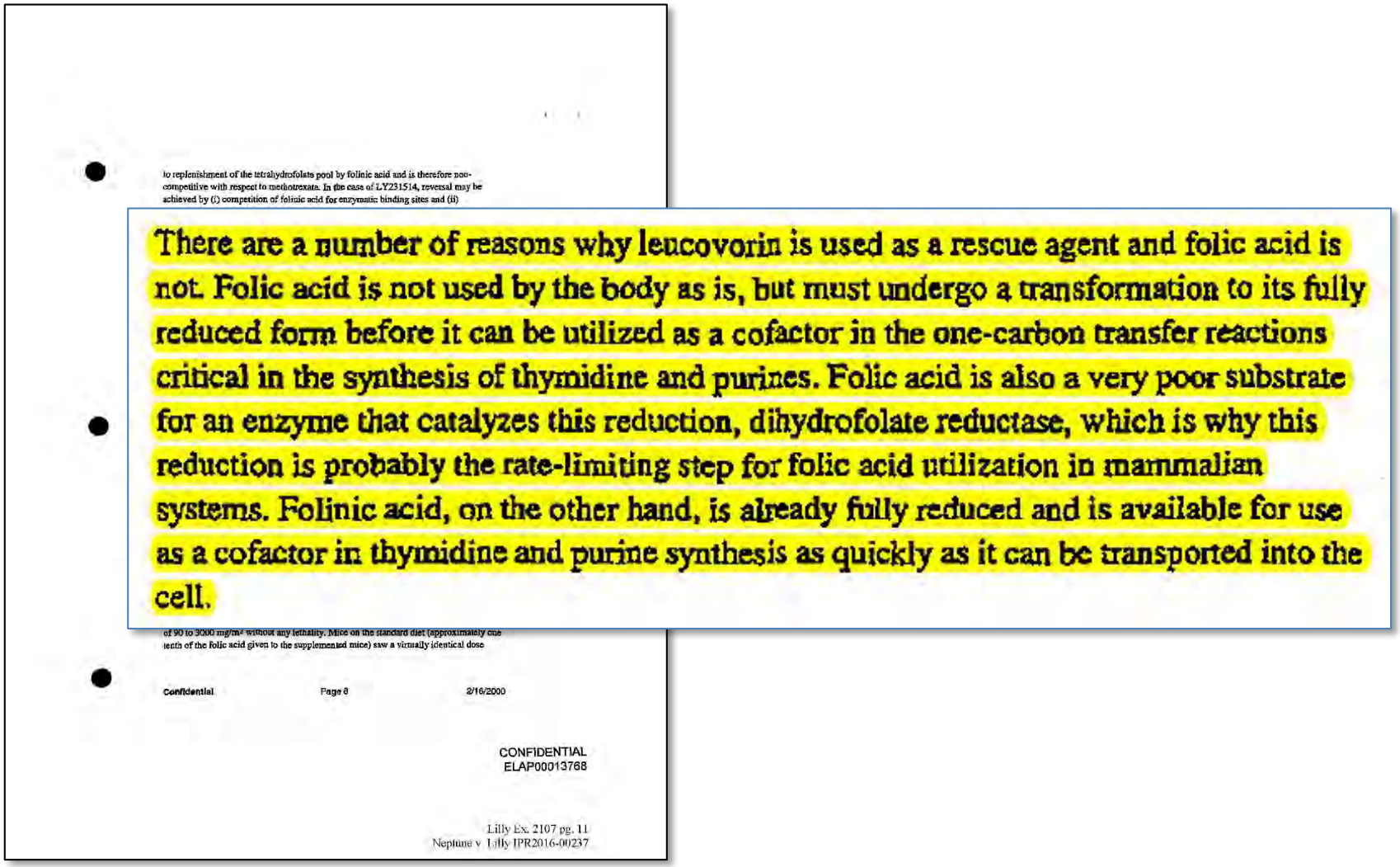

to replenishment of the tetrahydrofolate pool by folinic acid and is therefore non-competitive with respect to methotrexate. In the case of LY231514, reversal may be achieved by (i) competition of folinic acid for enzymatic binding sites and (ii)

There are a number of reasons why leucovorin is used as a rescue agent and folic acid is not. Folic acid is not used by the body as is, but must undergo a transformation to its fully reduced form before it can be utilized as a cofactor in the one-carbon transfer reactions critical in the synthesis of thymidine and purines. Folic acid is also a very poor substrate for an enzyme that catalyzes this reduction, dihydrofolate reductase, which is why this reduction is probably the rate-limiting step for folic acid utilization in mammalian systems. Folinic acid, on the other hand, is already fully reduced and is available for use as a cofactor in thymidine and purine synthesis as quickly as it can be transported into the cell.

of 90 to 3000 mg/m² without any lethality. Mice on the standard diet (approximately one tenth of the folic acid given to the supplemented mice) saw a virtually identical dose

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Source: Ex. 2107 at 11; Paper 48 at 13.

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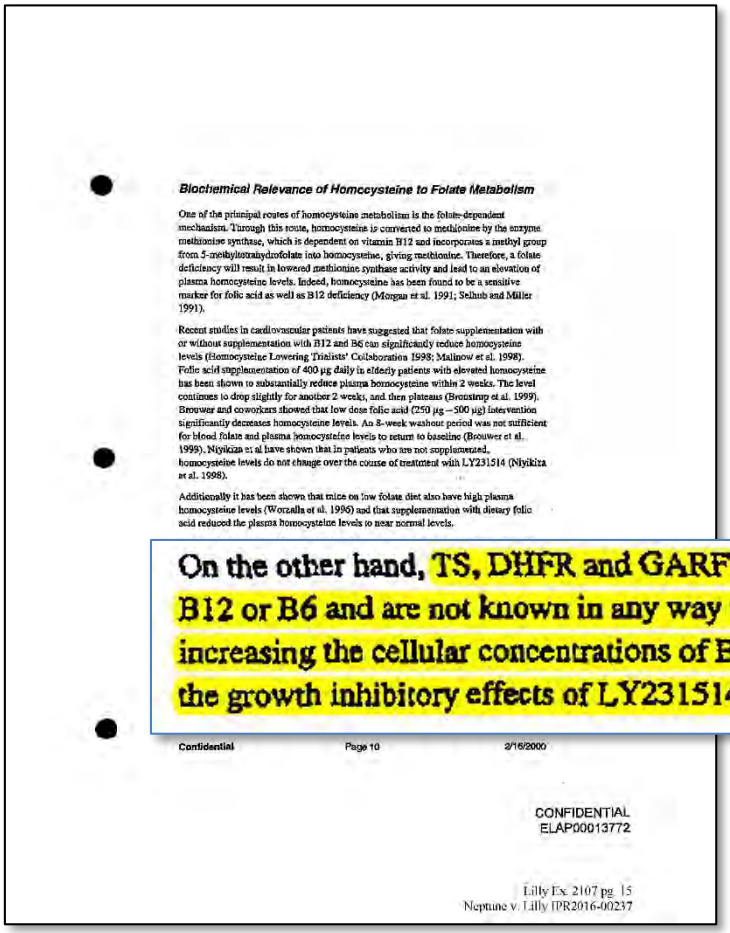

Biochemical Relevance of Homocysteine to Folate Metabolism

One of the principal routes of homocysteine metabolism is the folate-dependent mechanism. Through this route, homocysteine is converted to methionine by the enzyme methionine synthase, which is dependent on vitamin B12 and incorporates a methyl group from 5-methyltetrahydrofolate into homocysteine, giving methionine. Therefore, a folate deficiency will result in lowered methionine synthase activity and lead to an elevation of plasma homocysteine levels. Indeed, homocysteine has been found to be a sensitive marker for folic acid as well as B12 deficiency (Morgan et al. 1991; Selhub and Miller 1991).

Recent studies in cardiovascular patients have suggested that folate supplementation with or without supplementation with B12 and B6 can significantly reduce homocysteine levels (Homocysteine Lowering Trialists' Collaboration 1998; Malinow et al. 1998). Folic acid supplementation of 400 µg daily in elderly patients with elevated homocysteine has been shown to substantially reduce plasma homocysteine within 2 weeks. The level continues to drop slightly for another 2 weeks, and then plateaus (Bronstrup et al. 1999). Brouwer and coworkers showed that low dose folic acid (250 µg – 500 µg) intervention significantly decreases homocysteine levels. An 8-week washout period was not sufficient for blood folate and plasma homocysteine levels to return to baseline (Brouwer et al. 1999). Niyikiza et al have shown that in patients who are not supplemented, homocysteine levels do not change over the course of treatment with LY231514 (Niyikiza et al. 1998).

Additionally it has been shown that mice on low folate diet also have high plasma homocysteine levels (Worzalla et al. 1996) and that supplementation with dietary folic acid reduced the plasma homocysteine levels to near normal levels.

On the other hand, TS, DHFR and GARFT are not known to have any binding sites for B12 or B6 and are not known in any way to be affected by these two vitamins. Therefore, increasing the cellular concentrations of B12 and B6 is not expected to have an impact on the growth inhibitory effects of LY231514.

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Source: Ex. 2107 at 15; Paper 48 at 27.

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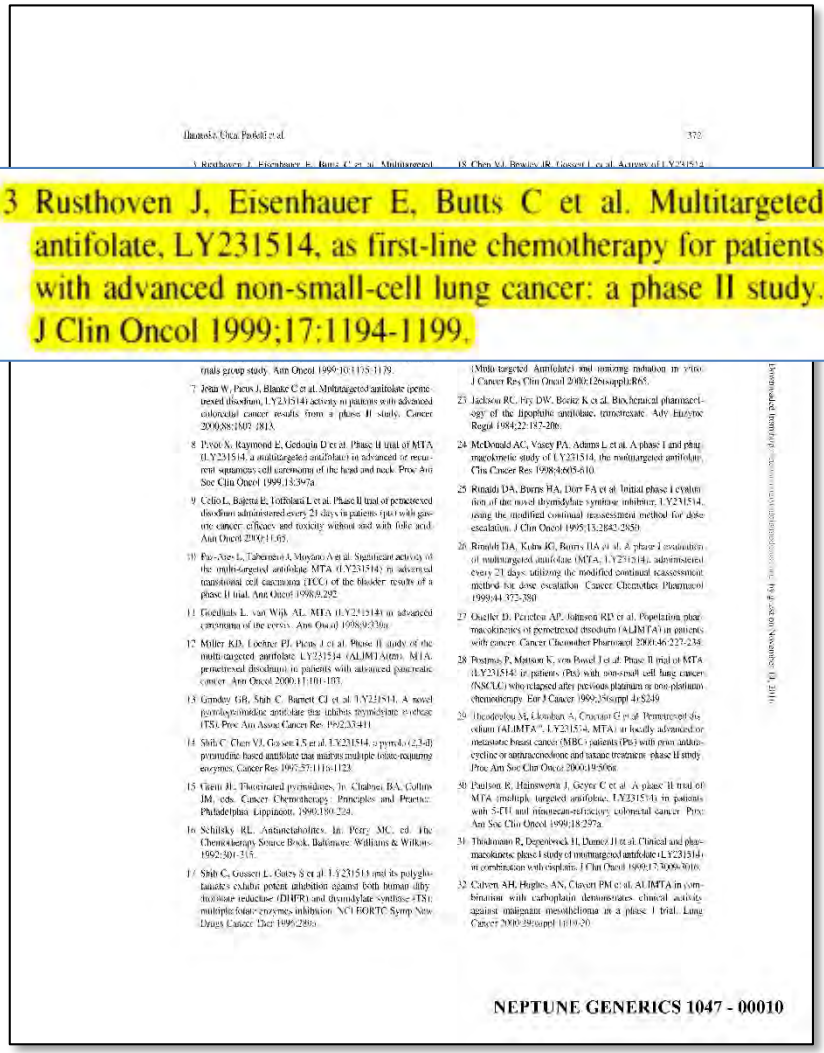
Oncologist Article



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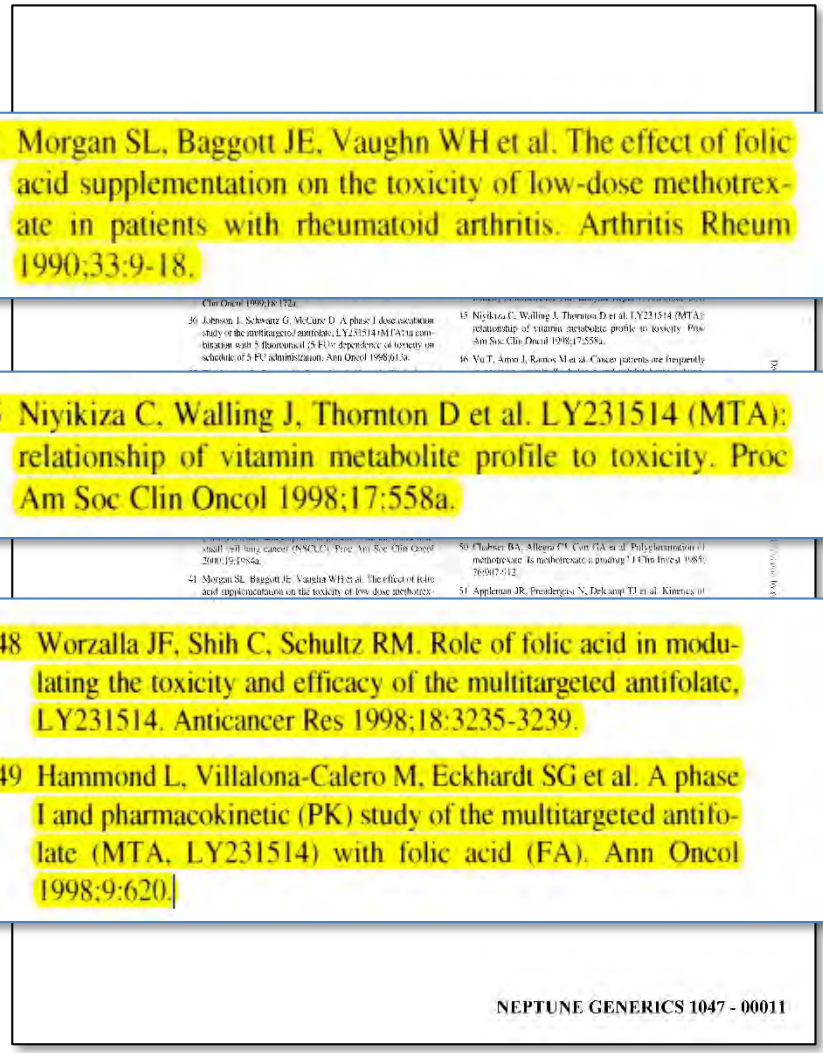

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DR. CHABNER

Dr. Chabner's Testimony

Q. My question is: Well, did you -- did you employ any other methodology other than your assumption that they would know the literature?

A. You know, I think that's giving me more credit than I'm due. I don't employ methodologies in defining POSA.

I -- I was asked the question: What would a person of ordinary skill in the art who was aware of all the relevant material in the public domain, what would

they know?

Q. What did you do to separate in your own mind what you know as a person of extraordinary skill, who won a lifetime achievement award 14 years before the date of invention?

A. Well, I didn't try to invent something that was -- what was less informed than myself, but I'm not sure what you expect me -- I really can't answer that question. I'm sorry.

Dr. Chabner's Testimony

Q. Does the person of ordinary skill in the art, the hypothetical person of ordinary skill in the art in this case, in your view, is it your opinion that that person would have 30 years of experience as an antifolate investigator?

A. You know, a person of ordinary skill is a legal definition of a person that doesn't exist. It's a person that knows everything that's in the public domain related to this topic. And it doesn't say that they have three years. They could learn it all in 10 days if they had to. And they could require it over 30 years.

My own personal experience as an

expert witness is based on my prior experience.

Source: Ex. 1075 at 228:11-229:3; Paper 48 at 2.

Dr. Chabner's Testimony

25 Q. And what -- how did you employ that
1 BRUCE CHABNER
2 standard in your analysis?
3 A. I looked at what I knew as -- as of
4 1999, and what the literature said and what
5 was available publicly, and I concluded that
6 it was not obvious that -- that using these
7 vitamins would make a difference, would
8 improve therapy.

Dr. Chabner's Testimony

A. I think, you know, it's like pornography. When it's reasonable, you understand it when you see it.

Scientifically, I was skeptical about it. So skepticism would mean not reasonable or obvious.

Source: Ex. 1075 at 89:20-25; Paper 48 at 2.

Dr. Chabner's Testimony

Q. So the prior art resulted in your skepticism. Is that -- is that your opinion?

A. That's right. The prior art that we quote here. I think the bottom line is I was skeptical about this working, based on

BRUCE CHABNER

my 30 years of experience as an antifolate investigator.

I wouldn't have tried it personally. I admire them for persisting and doing this and discovering that it actually did work, but I wouldn't have done it. And I think most people in the field wouldn't have done it. They didn't do it, in fact.

Q. Does the person of ordinary skill in the art, the hypothetical person of ordinary skill in the art in this case, in your view, is it your opinion that that person would have 30 years of experience as an antifolate investigator?

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My own personal experience as an

Dr. Chabner's Testimony

How reasonably successful did you think that vitamins plus pemetrexed needed to be in order to conclude not obvious?

MR. GROSSMAN: Objection to the form of the question.

A. I think it would have to be something that I could endorse. That I would say this

has a good chance of success.

And a lot of the stuff that was going into the clinic I thought had a very poor chance of success. So I wouldn't have endorsed most of that.

I know what your point is. Your point is that the success rate was low, but most of the stuff going into the clinic was not very good.

Q. What I --

A. So I would have been skeptical about most of it -- a lot of it, and I have a very good track record myself in terms of the drugs I put into the clinic, and I would endorse those as being -- having a reasonable chance of success.

If you would like to review what I've done personally, I can tell you what my -- my criteria were at the time. Included cisplatin, and carboplatin, and irinotecan, and fludarabine, and taxanes, and all of those were very successful drugs. And I think I have a very good track record of understanding what's -- what's got a good

Source: Ex. 1075 at 90:19-91:25; Paper 48 at 3.

Dr. Chabner's Testimony

BRUCE CHABNER

chance of success and reasonable chance and what doesn't.

And since that time, I have had a very good track record at Mass. General in putting things into the clinic, and I -- and I trust my own judgment about this.

When I'm skeptical about this, most of the time it doesn't work. I hope that answers your question.

Q. Is the standard that you just described that you personally apply and the success that you have had, is that what you had in mind for reasonable expectation of success when you conducted your analysis in your declaration --

MR. GROSSMAN: Objection to the form of the question.

A. Well, that's -- is that what -- you know.

Q. I will withdraw it and ask a better one.

A. I don't really get your point, you know. I think maybe you can explain it.

Q. I will withdraw it.

BRUCE CHABNER

When I asked you the question about what -- what the reasonable expectation of success needed to be on the realtime, you said, testified, I think it would have to be something that I would endorse. That would say that this has a good chance of success.

What did you mean by that?

MR. GROSSMAN: Objection to the form of the question.

A. I mean I looked at the scientific basis for what they're doing, what I knew about the drug, and I would say, you know, this -- I'm very skeptical or that this will work. And I haven't been wrong very many times.

And, on the other hand, with certain other drugs where I've looked at the data and I've -- I've endorsed the development of that drug, when I was at NCI this happened repeatedly, I think I had a track record of it.

So my -- my standard for saying what's obvious and reasonable is my personal

Source: Ex. 1075 at 92:1-93:25; Paper 48 at 3.

Dr. Chabner's Testimony

1	BRUCE CHABNER
2	standard. And that's why I have had 50
3	years in the field.

Dr. Chabner's Testimony

Q. What do you mean when you say there is no clinical result?

A. I mean there is no evidence that it worked in the clinical setting.

Q. And is evidence of something working in a clinical setting the criteria you employed to determine whether or not something would have a reasonable expectation of success?

MR. GROSSMAN: Objection to the form of the question.

A. Yes, because my -- my frame of mind was that it wasn't going to work, and this didn't prevent -- present any evidence to change that, and what was really needed was clinical evidence to change that mind -- my mindset about it.

BRUCE CHABNER

I'm an open-minded person, but I look -- I look for data that would convince me.

Source: Ex. 1075 at 214:9-215:4; Paper 48 at 3.

Dr. Chabner's Testimony

Q. And if I understand what you testified as to earlier, they would identify the high risk patients but then reduce the dose?

A. There are various ways of addressing this. One would be to reduce the dose and determine whether that had any deleterious effect on antitumor activity. Probably

wouldn't but might have.

They could use post treatment leucovorin if they felt it was folate related.

They could use G-CSF because the major toxicity they're concerned about is neutropenia, and we do that as a standard thing.

Q. Did they have any other options?

A. They had other options that I wouldn't have taken them.

Q. And in your declaration, the three options that you disclose are dose reduction, leucovorin and G-CFS, correct?

A. G-CSF.

Q. G-CFS, correct?

A. No. G-CSF.

Q. Those are the three, right?

A. Yes.

Granulocyte stimulating factor, G-CSF. Colony stimulating factor.

Yeah. I know it. We have our own lingo.

Q. Leucovorin rescue is -- that

Source: Ex. 1075 at 149:18-150:25; Paper 48 at 4.

Dr. Chabner's Testimony

BRUCE CHABNER

alternative that you've identified is --

A. Yeah.

Q. If I -- tell me if this is wrong because I'm not sure I fully appreciate it, but the idea there is you would have a patient that presented with high homocysteine. You would give them the drug anyway. They would -- it would result in toxicity and then you would give them a different drug to alleviate the fact that they have neutropenia, right?

A. So what -- my -- you're asking what I would have done.

Q. No. I'm asking -- I'm always asking what a person of ordinary skill in the art would have done.

A. A person of ordinary skill. I think the person of ordinary skill would have two choices. One to -- or three choices. One would be to try identify that group upfront by looking at homocysteine levels, and saying this is a higher risk group, I'm going to start them lower, and then dose escalate as they tolerate it. And that's

BRUCE CHABNER

standard in oncology, but I would study it to be sure that I was not compromising activity.

Dr. Chabner's Testimony

Q. Apart from escalating the dose above 500 or 600, if vitamins can increase the tolerability of the drug, would the person of ordinary skill in the art in June '99 understand that the vitamin supplements may permit -- may permit maintaining a -- a dose

rather than reducing the dose?

MR. GROSSMAN: Objection to the form of that question.

A. I understand your question, but that I think as of June of '99, one would be very concerned that they would also interfere with the antitumor activity of the drug. So you would think of other ways of dealing with this toxicity problem, as we've discussed extensively already today.

And so that's, you know, my answer is that ameliorating drug toxicity is fine as long as you're preserving antitumor activity, but if you're going to interfere with that, then you have -- there is no point in it.

Q. And if a person of ordinary skill in the art in June of 1999 was presented with a patient that had elevated homocysteine levels and was showing some toxicity at 500, what would be the options?

A. Reduce the dose in the next cycle, give them leucovorin after the first cycle and use G-CSF to -- to correct the

neutropenia.

Source: Ex. 1075 at 308:20-310:2; Paper 48 at 4.

Dr. Chabner's Testimony

Q. What data would have been available to the person of ordinary skill in the art in June 1999 to give them a reasonable expectation of success that reducing the dose to three-quarters for a patient that presented with high homocysteine levels would be either safe or efficacious?

A. They have other trials where they've done it. They had reduced the dose to 350 in a number of other trials when patients became toxic.

Q. And what data are you referring to that showed safety and efficacy at 350?

A. Well, they're Phase II trials and there's a whole bunch of them. They had the GI trials. They had the lung trials.

Q. And maybe we can go through them all, but can you identify one where the data showed efficacy and safety --

A. You know, I don't have the efficacy -- I don't think in any of these trials, we know what the efficacy was in patients who were -- were dose reduced, but I would say

from my prior experience in the field that if patients are toxic, that the toxicity would probably indicate toxicity for tumors as well.

So that dose reduction to a tolerable or well tolerated level would -- would still preserve the antitumor activity. And we have plenty of examples of that in cancer that have been worked out in the last 40 years.

So that would be my approach. You asked me what my approach would be.

Q. Actually, I asked you what the person of ordinary skill in the art would do.

A. Well, yes.

Dr. Chabner's Testimony

Q. And my question is: Can you identify a prior art reference where a dose reduction occurs for a high -- a patient that presented with high homocysteine levels?

A. No. But I can -- I can answer that

Source: Ex. 1075 at 105:8-12; Paper 48 at 5.

Dr. Chabner's Testimony

Q. How would the person of ordinary skill in the art decide to start with two-thirds or three-quarters of the dose?

A. Well, drug toxicity is related to -- to pharmacokinetic issues, such as duration of drug exposure, peak levels of drug. And the standard way of dealing with this is to just reduce the dose.

It depends on -- you know, I think it would have taken a little further study to see how much dose reduction would be necessary in these patients with homocysteine elevation. If, for example, I knew that there was evidence of renal dysfunction, I would probably cut the dose a little more than if I had no evidence of renal dysfunction, maybe to two-thirds of a dose or three-quarters.

Source: Ex. 1075 at 101:7-24; Paper 48 at 5.

Dr. Chabner's Testimony

problem, yes. And it's, you know, it's an issue to be further developed. I think that

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it would take further studies to establish exactly what is the well-tolerated dose in these -- in this situation.

Source: Ex. 1075 at 109:24-110:4; Paper 48 at 5.

Dr. Chabner's Testimony

My question is: Did the person of ordinary skill in the art in June of 1999 know what a tolerable dose would be for a patient that presented with elevated homocysteine levels?

MR. GROSSMAN: Objection. Form of the question.

A. I -- I don't think that the specific question was known, but this was a path to dealing with the problem and that's the path I would have taken.

Source: Ex. 1075 at 111:5-15; Paper 48 at 5.

Dr. Chabner's Testimony

A. My answer was -- my answer was that was the path I would have taken to study this issue. So you would get -- do a study in terms of if I dose reduce these patients to 350, would I avoid the toxicity? And I can easily measure homocysteine before I

treat and I can do that. I could do the study.

I don't think that there -- you know, the information wasn't complete at that time, but that's the path I would have

Source: Ex. 1075 at 112:20-113:6; Paper 48 at 5.

Dr. Chabner's Testimony

Q. What is -- what data are you aware of that would have given the person of ordinary skill in the art confidence in June of 1999 that three-quarters of a dose in a high homocysteine patient would not result in toxicity?

A. I -- well, there are two things. One is 70 percent of the patients who became toxic had high homocysteine. So you relate

that to other trials where they were dose reduced, and they were able to continue to treat those patients at 350 after they became toxic. So I assume at least a portion of those patients had high homocysteine.

Q. You don't know that, do you? Is there any data?

A. Do I know that?

Q. Yeah.

A. There is a lot of things I don't know, but I think that's a fair assumption.

Dr. Chabner's Testimony

that I would be very encouraged to undertake further investigation, and I would pay attention to the issue of whether dose reduction was associated with a decrease in tumor response but --

Q. Does being encouraged to undertake further investigation satisfy your understanding and application of a reasonable expectation of success?

MR. GROSSMAN: Objection to the form of the question.

A. Yes.

Source: Ex. 1075 at 136:2-13; Paper 48 at 5.

Dr. Chabner's Testimony

Q. Were you the first editor-in-chief?
A. I was.
Q. That was in 1994?
A. You know, I can't tell you the exact date. Probably in the early '90s.
Q. Are you still the editor-in-chief of The Oncologist?
A. I am.

Source: Ex. 1075 at 34:13-20; Paper 48 at 6.

Dr. Chabner's Testimony

BRUCE CHABNER

A. That is a statement of fact that maybe one patient was treated at 925. The conclusion of it was that that was not the dose -- they could bring to the clinic. I think you're aware of that.

Since we are talking post facto, what was the dose used clinically? It was 500, right? So your argument is full of holes here. You wouldn't -- you wouldn't use 925 in a patient. If you did, you would be sued; am I correct?

Q. This seems to be in conflict with your opinion that a person of ordinary skill in the art would have taken from Hammond failure?

MR. GROSSMAN: Objection to the form of the question.

Q. Does it not?

A. What -- yes. That -- my opinion is that people would look at the abstract and say it failed.

Q. Did your reviewers fail here by letting this through?

A. Pardon?

BRUCE CHABNER

Q. Did your reviewers fail by letting this through?

A. What do you mean, did my reviewers fail? What a stupid question. Come on.

Q. Well how -- I mean you would agree that that --

A. I don't read -- I don't read every sentence in every paper that is published in that journal, do I?

Q. Do you wish you would have so you could have corrected --

A. I wish I would have corrected it, yes, sir.

Source: Ex. 1075 at 147:1-148:14; Paper 48 at 6.

Dr. Chabner's Testimony

Q. And when you said that this paper,

BRUCE CHABNER

Exhibit 1047, is pretty straightforward, what do you mean by that?

MR. GROSSMAN: Objection to the form of the question.

A. Well, it was -- it was an established drug at this point. People knew about it. They knew about its clinical activity. They knew about its pathway and it wasn't something that was really, you know -- it wasn't very exotic like gene therapy of a brain tumor with a viral vector. This is -- this is bread-and-butter oncology right here.

Q. Way less complicated that gene therapy of a brain tumor with a viral vector. Is that what you're saying?

A. Well, not -- I wouldn't say less complicated but less -- you know, more established as a way of treating cancer.

There are lots of wacky ideas out there, some of which end up working, but -- which are controversial and, you know, you have to be, you know, circumspect about publishing things like that.

BRUCE CHABNER

Q. And Exhibit 1047 was not one of those kinds of wacky controversial papers? Is that what you're saying?

A. Well, it's -- I wouldn't say the paper is not --

MR. GROSSMAN: Objection.

A. -- I mean controversial, but it's -- it's bread-and-butter oncology. This is cancer pharmacology. This is cancer clinical trials.

And we had, you know, prior experience with antifolates. This is a new antifolate. We knew how to read the paper and -- and understand it. If somebody asked me to take a paper of viral gene therapy vectors, I would have a hard time understanding the vector and how it actually worked, but this is -- this is bread-and-butter pharmacology.

Source: Ex. 1075 at 82:25-84:20; Paper 48 at 7.

Dr. Chabner's Testimony

Q. What did the person of ordinary skill

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in the art as of June of 1999, what would that person take from Hammond, from the Hammond disclosure?

A. A person of ordinary skill would see the very poor response rate. The fact that -- the logic of the trial was that they could dose escalate with the drug and get a better response rate. That proved not to be the case.

They began running into renal toxicity, which is a serious problem for a drug that undergoes renal clearance. So they couldn't dose escalate. I mean with methotrexate you can go up tenfold, even more in dose. They couldn't do that. And so they were -- the result was that they had one response in the 30-some patients that they studied.

Dr. Chabner's Testimony

Q. Read for me, please, Dr. Chabner, the sentence --

A. I have read it.

Q. -- that is referenced -- that has reference 49 that was published in the journal you are the editor-in-chief of.

Will you please read that sentence.

A. "While the trial is still currently ongoing, preliminary results indicate the addition of folic acid ameliorates toxicity permitting dose escalation of pemetrexed up to at least 925 milligrams per milliliter in heavily pretreated patients."

Q. And where did they get that from?

BRUCE CHABNER

A. That is a statement of fact that maybe one patient was treated at 925. The conclusion of it was that that was not the dose -- they could bring to the clinic. I think you're aware of that.

Since we are talking post facto, what was the dose used clinically? It was 500, right? So your argument is full of holes here. You wouldn't -- you wouldn't use 925 in a patient. If you did, you would be sued; am I correct?

Q. This seems to be in conflict with your opinion that a person of ordinary skill in the art would have taken from Hammond failure?

MR. GROSSMAN: Objection to the form of the question.

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BRUCE CHABNER

Q. Did your reviewers fail by letting this through?

A. What do you mean, did my reviewers fail? What a stupid question. Come on.

Q. Well how -- I mean you would agree that that --

A. I don't read -- I don't read every sentence in every paper that is published in that journal, do I?

Q. Do you wish you would have so you could have corrected --

A. I wish I would have corrected it, yes, sir.

Source: Ex. 1075 at 146:11-148:14; Paper 48 at 9.

Dr. Chabner's Testimony

Q. The next paragraph starting with "ongoing LY231514 trials." That's where I am next. It says, "Ongoing LY231514 trials include a Phase I study of LY231514 and folic acid. An interim report suggests that folic acid supplementation in this study permits dose escalation by ameliorating toxicity since heavily and minimally pretreated patients tolerate LY231514 at doses of 700 and 925, respectively. Reference 10."

Do you see that?

BRUCE CHABNER

A. I see that.

Q. What is reference 10 --

A. That's --

Q. -- that is cited for that proposition?

A. That is a Hammond paper. Not the abstract. Where do we have the list of the references?

Q. Reference 10, if you go back. It's abstract 620, correct?

A. I don't see it. Annals of oncology supplement 4. Yes. I guess that's the same. I -- I'm not sure. I guess it is the same abstract that we've looked at.

Q. Yes. That reference 10 if you -- I think you have it in front of you.

A. Yeah.

Q. Reference 10 is Exhibit 1022 in this proceeding.

A. Okay. All right.

Source: Ex. 1075 at 167:14-168:21; Paper 48 at 9.

Dr. Chabner's Testimony

Q. Sure. So we are on Exhibit 2035, which is a Hammond abstract.

A. Yes, right.

Q. And the last sentence of that abstract concludes, "These results indicate that folic acid supplementation appears to permit MTA dose escalation."

Do you see that?

A. It does.

Q. And would a person of ordinary skill in the art as of June of 1999 have any reason to disagree with the conclusion of Hammond?

BRUCE CHABNER

A. Yes, they would, because we knew that there was a problem with creatinine clearance in these patients.

Q. And have you cited or identified anywhere, Dr. Chabner, any publication in the prior art or otherwise that takes issue with the Hammond conclusion?

A. Oh, my God. Why would -- I mean why would they argue with it? I don't think they would. I mean that is his conclusion. He is entitled to make his conclusion. He did dose escalate. It doesn't say that that was the dose they chose -- they could choose to study further.

They didn't. I mean I think it's obvious that as of June 1999, they weren't studying 900 milligrams per meter squared with folate supplementation, unless I'm mistaken.

Q. Does Hammond's abstract state or imply to a person of ordinary skill in the art anything that was disappointing about the results of their study?

A. No. Of course, the fact that they had

Source: Ex. 1075 at 180:13-181:25; Paper 48 at 9.

Dr. Chabner's Testimony

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one response was disappointing. If they had seen 10 responses, they would have jumping for joy.

I wasn't there at this presentation so I don't know what -- what his feelings were about it.

Q. Would a person of ordinary skill in the art as of June 1999 understand from this Hammond abstract that folic acid may permit a patient to take either additional cycles or a greater dose than they might otherwise tolerate?

A. I think what a person of ordinary skill would know in June of 1999 is that folate supplementation was not associated with what appeared to be an improvement in response rates, and it may -- the data were unclear as to how much of a dose escalation was permitted because of this -- this problem with impairment of creatinine clearance.

And so they -- you know, it didn't work out that they would use dose escalation with folate supplementation.

BRUCE CHABNER

Q. Would --

A. It never did.

Q. Sorry. You finished?

A. They never did.

Q. Would a person of ordinary skill in the art as of June 1999 understand from Hammond that folic acid may permit a patient that had to go from a 500 to a 350 dose could remain at the 500 amount if it was accompanied with folic acid pretreatment?

A. That really wasn't proven, but, you know, it is still possible. And I think that what we don't know is that if that folate was added, whether that would reverse the antitumor activity. That was still in question.

Q. Well, we know it didn't completely reverse the antitumor activity from Hammond, don't we?

A. That's one patient.

Q. So is that a yes?

A. There was one patient that responded.

Q. And so we know that the administration of folic acid in Hammond did

Dr. Chabner's Testimony

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not eliminate antitumor activity --

A. Did not completely eliminate, yes.

Q. In fact, not only did it not completely eliminate it, there was a patient who took it and got a partial response, correct?

A. Well, that's what I mean by did not completely eliminate it. We may have 90 percent eliminated it, but we don't know. But it certainly wasn't an encouraging result, you know.

Q. Your opinion is that Hammond was not an encouraging result?

A. Not at all.

Source: Ex. 1075 at 184:1-15; Paper 48 at 9.

Dr. Chabner's Testimony

Q. So you disagree with everyone who thought Hammond showed some encouraging signs towards folic acid pretrial?

A. I disagree with everyone. I'm just stating what I think that a person of ordinary skill would conclude from happening. I think a lot -- you know, there was not -- also a prior trial at Lilly with lometrexol which -- which showed the same negative result.

So I think it was a discouraging. I mean it was virtually the same result. It was one response in 30-some patients, and they -- they dropped lometrexol. So it didn't seem to me that was a promising avenue of approach.

Source: Ex. 1075 at 160:5-20; Paper 48 at 9.

Dr. Chabner's Testimony

In preparing your declaration for the two or three years you've been working on this matter --

A. Yeah.

BRUCE CHABNER

Q. -- and studying it, have you ever identified anyone, any author in the prior art or after the prior art that characterizes Hammond like you do?

A. You know, I haven't asked that question of any people, I'm sorry to say. I would say this, that the company didn't -- didn't use that regimen ever again.

Q. The company didn't use that regimen. The company pretreated, used folic acid.

A. They didn't use that regimen. The

Dr. Chabner's Testimony

Q. Turn back to Exhibit 1047, which is The Oncologist reference.
A. Okay.
Q. If you turn to Page 371.
A. 371. Where is that?
Q. Table 5 is at the top of 371.
A. Wait a minute. Just a second. Got it.
Q. The last sentence before the first full paragraph that starts with "concurrent."
Are you with me?
A. Yes.
Q. Exhibit 1047, that sentence says, "Concurrent with this analysis, preclinical data supported the notion that oral folate supplementation markedly reduced toxicities in mice while maintaining antitumor efficacy, reference 48."
Do you see that?
A. I see that.

BRUCE CHABNER
Q. And what is reference 48?
A. It's probably Dr. Worzalla, or Mr. Worzalla I should say.
Q. Worzalla is attributed for that statement?
A. Yes.
Q. Do you disagree?
A. I think if you take each element of it, you can -- you can say he did show this. He showed that you could reduce toxicity by giving the drug -- folic acid. That's not an issue. The issue is do you get a better therapeutic result.
You get the same therapeutic result essentially until you get -- the only data that -- in that abstract that shows any possible advantages is at doses which you could never achieve in people, never.
Q. Do you think this is a wrong -- an inaccurate citation to Worzalla?
MR. GROSSMAN: Objection to the form of the question.
A. Is it inaccurate?
MR. GROSSMAN: Dr. Chabner. I'm

Source: Ex. 1075 at 157:5-158:25; Paper 48 at 10.

Dr. Chabner's Testimony

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going to restate my objection, Counsel. This is like the third or fourth time. This document is not prior art. It's improper what you're doing, and I object and reserve the right to move to exclude all the testimony about this document.

MR. SKIERMONT: Noted.

Q. Do you think this wrong, miscited?

A. I don't think it's absolutely correct, that's right. And I was the editor, but I don't read every paper. And I don't endorse every sentence in every paper in my journal. There is certain freedom that people have to make statements, which they're -- it's fine with me if, you know, they interpret it a different way.

But that's my interpretation. I think that a person of ordinary skill would have the same interpretation.

Source: Ex. 1075 at 159:1-20; Paper 48 at 10.

Dr. Chabner's Testimony

Q. And do you understand, Dr. Chabner, that Lilly Exhibit 2103 is a Lilly submission to the FDA where Lilly is telling the FDA or -- or suggesting to the FDA that they want to add vitamins to the pemetrexed --

A. Yes.

Q. -- regimen, correct?

A. I do.

Q. Now, you will see at the end of this brief, before the page that says "Dear JCMH Investigator."

A. I don't.

Q. And I can turn it for you.

A. Yeah. Show me what you're talking about.

(Document tendered.)

Q. At the end of that brief, there is a section called "references."

A. Gotcha.

Q. And do you see that reference 9 is

Worzalla, which is Exhibit 1005 in our proceeding. And reference 10 is Hammond, which is Exhibit 1022 in our proceeding.

Do you see that?

A. I see them both.

Q. Now, if you would turn back to the -- to the brief, which is Page 3 of 20. It says that in the bottom left-hand corner.

A. Got it.

Q. There is a -- first full paragraph on Page 3 is a sentence that starts "as with lometrexol."

A. Yes.

Q. And if you could read that paragraph.

A. "In vivo experiments with, I guess, pemetrexed have suggested that supplemental folate modulates its toxicity profile in antitumor activity. The LD 50 of the drug occurred at 60 to 200-fold lower doses of this drug in DBA/2 and CD1 nu/nu mice maintained on a low folate diet compared with those fed standard diets. In these experiments the antitumor activity of the drug was preserved."

Source: Ex. 1075 at 165:5-166:25; Paper 48 at 10.

Dr. Chabner's Testimony

BRUCE CHABNER

Q. And the drug that they're talking about that was, the antitumor activity was preserved is LY231514?

A. That's correct.

Q. And that's pemetrexed?

A. Right.

Q. And the reference cited to substantiate the argument that Lilly was making to the FDA is reference 9?

A. Yes.

Q. And what is reference 9?

A. Worzalla.

Source: Ex. 1075 at 167:1-13; Paper 48 at 10.

Dr. Chabner

Dr. Chabner's Testimony

Q. And then it goes on, "Concurrent with this analysis, preclinical data supported the notion that oral folates supplementation

markedly reduce toxicities in mice while maintaining antitumor efficacy," and that cites to Worzalla prior art, correct?

A. Yes. And as I pointed out previously, you take apart that sentence, it certainly did reduce toxicities. It shifted the curve, the dose-response curve far to the right. And you did get the same efficacy but at a much higher dose, and that begins to be a problem -- not begins to be. It confronts a problem, and that is, you can't escalate the dose of pemetrexed indefinitely in patients.

So the relevance of this is questionable.

Q. And did you raise any of these issues at the time that this article --

A. I didn't -- I don't believe --

Q. -- was published in the journal --

A. I don't believe I reviewed the paper --

Q. -- that you're editor --

A. I don't think I reviewed this paper in detail. I thought it was worth publishing,

Source: Ex. 1075 at 219:23-220:25; Paper 48 at 10.

Dr. Chabner's Testimony

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obviously. It got published but, you know, I don't -- I don't conduct a deposition with each of my authors, if that's what you're saying.

Source: Ex. 1075 at 221:1-5; Paper 48 at 10.

Dr. Chabner's Testimony

Q. "Because tumor issue and normal tissue such as" -- sorry -- "because tumor tissue and normal tissue, such as bone marrow, presumably have different folate requirements, it is possible to decrease the toxicity to healthy tissue while maintaining antitumor effect through careful adjustment of folic acid intake. This has been shown in experimental systems for LY231514 and another antifolate, lometrexol," citing to Worzalla and Alati.

Do you see that?

A. I do.

Q. And is the -- and, Worzalla, is that

the same -- is that the same Worzalla that we've been -- we have looked at today?

A. Yes.

Q. Worzalla 1998, correct?

A. Right.

Q. And so Lilly told the FDA that the prior art Worzalla document showed that LY -- has shown for LY231514 that it is possible to decrease the toxicity to healthy tissue while maintaining antitumor effect through careful adjustment of folic acid intake."

MR. GROSSMAN: Objection.

Q. Do you agree with that?

MR. GROSSMAN: Objection to the form of the question.

A. I, you know, I wouldn't have said that. No, I don't agree with that. I'm sorry.

Dr. Chabner's Testimony

Q. All right. So I want to go to Page 12, the second page of this discussion. And do you see that the data -- there is a cite Worzalla et al. 1998.

A. Yes.

Q. Do you see that that's the Worzalla --

A. Now, wait a minute. What page are you going to?

Q. Page 12, the next page right above the table.

A. Wait a minute. Page -- yeah. We are switching pages here unfortunately. Okay.

Q. Okay. You with me?

And you see where it says Worzalla et al. 1998?

A. Yeah.

Q. And that's the same Worzalla that's at issue in this case, correct?

A. Right.

Q. And there is a table and the sentence or the paragraph below the table Lilly wrote to the FDA, "These data" -- and this is a

reference to prior data --

A. Right.

MR. GROSSMAN: Objection to the form of the question.

Q. "These data show that antitumor activity is virtually identical in mice receiving a standard diet to that in mice receiving a 10-fold increase in daily folic acid. Mice receiving the extra folic acid also showed a decrease lethality at higher doses of LY231514. These data support the hypothesis that folic acid supplementation can protect healthy tissue from the toxic effects of LY231514 with retention of antitumor activity."

Do you see where Lilly --

A. I do see that.

Q. -- said that to FDA?

A. Yeah. Right.

Q. Do you think Lilly misled the FDA about what the prior art did --

A. I don't think they -- I'm not.

MR. GROSSMAN: Objection to the form of the question.

Dr. Chabner's Testimony

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A. I'm not in a position to judge whether they misled it.

My interpretation of this is, first of all, the standard diet is a high folic diet. It's not the human diet. So this moves the doses way up.

Then they add a much larger increment of folate with a low folate plus 15 milligrams per kilogram.

Do you realize for a human being that would be like 60 to 100 milligrams of folate a day. We wouldn't take that. It would turn our urine like iridescent yellow.

And at doses of maximum activity -- I'm looking at the table. I hope you can see that. 90 to 3,000 milligrams per meter squared.

I mean there is no way you could give that on a daily basis to an animal -- a human and even come close to those doses.

I mean this is irrelevant. You're dealing with a mouse that has a creatinine clearance like 50 times that of a human. He is able to cure -- eliminate the drug fast

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and has a tolerance for drugs which is much greater than humans.

So I don't know how to interpret that data. I don't think it's -- it tells me that I can do this in people because I can't get to those kinds of doses.

The 3 -- 3 grams per meter squared in a human would be like 12 grams per meter squared -- 3 grams per meter squared in a mouse would be in the range of 12 grams per meter squared in humans.

There is no way you can do that. I mean you're starting to get creatinine changes at 600.

So, you know, I think they interpreted the experiment accurately, and what they said in this experiment, with this tumor, a very disabled mutant tumor, which doesn't resemble any human tumor I've ever seen, and with these high doses in mice and with this extremely, extremely high folate supplementation, they can actually get away with these high doses. And they may have seen a broader window for the therapeutic

Source: Ex. 1075 at 271:1-272:25; Paper 48 at 10.

Dr. Chabner's Testimony

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ratio, yes.

But, you know, whether this could be done in people, I think it's highly, highly dubious.

Q. Would a person of ordinary skill in the art in June of 1999 disagree -- agree or disagree that the Worzalla '98 data support the hypothesis that folic acid supplementation can protect healthy tissue from the toxic effects of LY231514 with retention of antitumor activity?

MR. GROSSMAN: Objection to the form of the question. I would renew my objection.

A. I would look to the Hammond trial. I wouldn't -- I would say, "Look, this is a really weird regimen in mice with a disabled tumor." I would look to the human trials to see what that says.

In the human trials Hammond showed that you couldn't escalate. I mean you couldn't go to -- from 500 to 5,000 milligrams per meter squared like they did in the mouse. And, you know, you're using

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once -- a one-day regimen rather than a constant daily regimen. And you're treating human tumors. You're not treating this disabled poor tumor that they used in mice, which has, you know, a hypersensitivity to TS inhibitors.

So I would say, "Show me the human data. What did it do in people?" And I found that, you know, totally unconvincing that I should go forward with it.

Now, why they did this with the FDA, I don't know. Things happened between 1999 June and when this document went in. They had their reasons for doing it. I'm not privy to that. I suspect it was that their regimen was very toxic, that they were testing a mesothelioma and they were eager to find a way to keep going. They had a big investment in this so they did it.

MR. GROSSMAN: Counsel --

A. They used a regimen which had never been tried in people, as far as I know. And that was folic acid and B12. I've never seen any paper prior to '99 where that

Source: Ex. 1075 at 273:1-274:25; Paper 48 at 10.

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combination was used to ameliorate pemetrexed toxicity.

So their reasons for doing this and their rationale for suddenly changing their trial, I think that's their business.

Q. Just to be clear --

MR. GROSSMAN: Counsel, I'm going to renew my objection about this document. It's not prior art. It's an Eli Lilly document.

Q. Just to be clear, so I understand your opinion --

A. Yeah.

Q. -- your opinion, Dr. Chabner, is that a person of ordinary skill in the art in June of 1999 would conclude that the Worzalla data does not support the hypothesis that folic acid supplementation can protect healthy tissue from the toxic effects of pemetrexed with retention of antitumor activity; is that right?

MR. GROSSMAN: Objection to the form of the question.

A. I would -- I would say because you

BRUCE CHABNER

have the Hammond trial, you have data in people. You have to specify whether you're talking about a mouse with this tumor or with people. And if you are asking me to support that statement, I would say in Worzalla's mouse it seemed to work, at the very highest dose, in a dose which is not achievable in people.

And you can't point out to me any instance where they've gone even close to these doses in people.

Q. So your opinion is that a POSA in June 1999 would conclude that the Worzalla data does not support the hypothesis that you can have antitumor activity and reduced toxicity?

A. My -- I -- you say it was in 1999. In 1999, I know Hammond. And so I have doubts about this data, yes.

Q. And when Lilly wrote to the FDA that the data support that hypothesis, they, of course, knew about Hammond too, didn't they?

A. You know, I'm not sure exactly what they -- what they told the FDA. This is not

BRUCE CHABNER

part of my testimony.

Source: Ex. 1075 at 275:1-277:22; Paper 48 at 10.

Dr. Chabner's Testimony

Q. And are you aware of any publication that sides with your interpretation of Worzalla and not the one that is disclosed in The Oncologist article --

A. No.

Q. -- or these FDA reports?

Source: Ex. 1075 at 251:19-24; Paper 48 at 10.

Dr. Chabner's Testimony

Q. And so we were -- before the break, we were talking about Worzalla and the reference to Worzalla here.

And, in addition, this paragraph also communicates -- is Lilly communicating to the FDA that it is possible to decrease the toxicity to healthy tissue while maintaining antitumor effect through careful adjustment of folic acid -- folic acid intake.

Another -- other reference is cited as "Clinical Trials with Lometrexol," citing Young et al. in 1992 and Laohavinij.

A. "Laohavinij."

Q. Laohavinij?

MR. GROSSMAN: Laohavinij.

Q. Laohavinij in 1996.

Do you see that?

A. I do.

Q. And do you understand in reading this that Lilly is presenting an argument to the FDA that its experience with folic acid and lometrexol in clinical trials supports

Lilly's idea that you can preserve the antitumor effect while reducing toxicity by administering folic acid with an antifolate?

MR. GROSSMAN: Objection to the form of the question.

A. That was their statement in this -- this paragraph. I don't agree with that statement. My reading of those papers is different. I think we've gone into that.

Q. And just to be clear, you think that Lilly was mistaken when they told the FDA that the clinical trials with lometrexol supported the proposition they are citing Young and the Laohavinij article for?

A. That -- that they could decrease toxicity while maintaining antitumor activity, I -- I haven't -- I haven't seen data to support that statement in Laohavinij.

And as far as I know, Worzalla and the other guy, Alati, are preclinical studies, and we -- we can go over those in some more detail, but I don't agree that that's my conclusion.

Source: Ex. 1075 at 254:3-255:25; Paper 48 at 10.

Dr. Chabner's Testimony

BRUCE CHABNER

A. My reading of those articles does not -- I wouldn't have said that based on my reading of those articles.

Q. And does your declaration, Dr. Chabner, cite any published literature before or after June of 1999 that agrees with your interpretation of the lometrexol clinical trials as opposed to Lilly's?

A. Subsequently?

Q. Either -- anytime. Anytime in the history of the world.

A. Well, I'm not prepared to testify about anything after 1999. I'm sorry.

Q. My question was -- that answers my question. You didn't look for anything after 1999.

Are you aware of anything prior to 1999 that has been published where a peer-reviewed article concludes that the clinical trial experience with lometrexol doesn't -- do not demonstrate decreased toxicity while maintaining antitumor effect?

A. Well, I think it was obvious. I'm not aware of everything that was published

BRUCE CHABNER

A. My reading of those articles does not -- I wouldn't have said that based on my reading of those articles.

Q. And does your declaration, Dr. Chabner, cite any published literature before or after June of 1999 that agrees with your interpretation of the lometrexol clinical trials as opposed to Lilly's?

A. Subsequently?

Q. Either -- anytime. Anytime in the history of the world.

A. Well, I'm not prepared to testify about anything after 1999. I'm sorry.

Q. My question was -- that answers my question. You didn't look for anything after 1999.

Are you aware of anything prior to 1999 that has been published where a peer-reviewed article concludes that the clinical trial experience with lometrexol doesn't -- do not demonstrate decreased toxicity while maintaining antitumor effect?

A. Well, I think it was obvious. I'm not aware of everything that was published

Source: Ex. 1075 at 256:1-257:25; Paper 48 at 10.

Dr. Chabner's Testimony

Q. That wasn't a better antifolate as of June of 1999 than pemetrexed, was there?

A. There was one in development and it turned out to be better for -- for another subset of tumors.

Q. Which one was that?

A. Pralatrexate.

Q. And the -- there was one that was in development that as of June of 1999, the person of ordinary skill in the art would not have known it was superior to pemetrexed, correct?

A. Absolutely right.

Source: Ex. 1075 at 153:2-14; Paper 48 at 12.

Dr. Chabner's Testimony

17 A. Therapeutic index is a -- is a
18 relationship between activity and toxicity,
19 and what you're looking for is a window in
20 which there is a therapeutic response and
21 tolerable toxicity.

Source: Ex. 1075 at 184:17-21; Paper 48 at 15.

Dr. Chabner's Testimony

In a Phase I trial, you're just trying to find a safe and effective dose to carry on. So there is a big difference

Dr. Chabner's Testimony

Your -- your question is: Would I judge that that is statistically significantly different? It's not a randomized trial so you really can't do that kind of statistical comparison.

It's unlikely to be a significant difference, I think statistically, but it's certainly not an improvement.

Q. It's about 50/50, right?

A. Pardon?

Q. The P -- the Chi-Square P-value would be just a little bit less than 50/50?

A. You couldn't do a Chi-Square on that kind of data.

Q. Oh, you don't want to compare --

A. Well, you look at them both. You look at them, sure.

But it's not a randomized trial, so you can't do that. I mean I wouldn't do it scientifically. It wouldn't be valid. Different patients. Diff --

You know -- you know, you would just have to assess what you know about Phase I trials and say which is the

promising way to go.

Q. You said it wouldn't -- you said different patients. There are different patients in these two -- in Hammond and Rinaldi?

A. Well, they are Phase I patients, but they are not -- they are selected from the same patient group. That's right.

Q. And they have different diseases, too, don't they?

A. They have a variety of diseases. That's right.

Source: Ex. 1075 at 178:2-179:13; Paper 48 at 16.

Dr. Chabner's Testimony

Q. You said it wouldn't -- you said different patients. There are different patients in these two -- in Hammond and Rinaldi?

A. Well, they are Phase I patients, but they are not -- they are selected from the same patient group. That's right.

Q. And they have different diseases, too, don't they?

A. They have a variety of diseases. That's right.

Q. Do we know whether they received the same doses of the drug?

A. I'm not -- no, they didn't receive the same doses of the drug. I made that point earlier.

Source: Ex. 1075 at 179:3-18; Paper 48 at 17.

Dr. Chabner's Testimony

Q. Was it known to a person of ordinary skill in art as of June of 1999 that homocysteine is a sensitive marker for folic acid as well as B12 deficiency?

A. Well, I think what was known is that homocysteine elevations were found in folic acid/B12 deficiency, and that at least a subset of those patient had either folic acid deficiency or B12, but it didn't distinguish between the two until you did methylmalonic acid assessment.

Source: Ex. 1075 at 280:10-20; Paper 48 at 19.

Dr. Chabner's Testimony

He showed that you could reduce toxicity by giving the drug -- folic acid. That's not an issue. The issue is do you get a better therapeutic result.

Source: Ex. 1075 at 158:11-14; Paper 48 at 22.

Dr. Chabner's Testimony

9	trap, the issue of methyl trap is very rare.

Dr. Chabner's Testimony

So I would say, "Show me the human data. What did it do in people?" And I

Dr. Chabner's Testimony

13 You have to look at the data.

3 look -- I look for data that would convince
4 me.

8 So I would say, "Show me the human
9 data. What did it do in people?" And I

Dr. Chabner's Testimony

Q. Does the patent anywhere disclose why it is important to select those doses?

A. The patent sort of gives some flexibility to the physician, but I can tell you in practice everybody uses 1000.

Source: Ex. 1075 at 352:5-9; Paper 48 at 30.

Dr. Chabner's Testimony

Q. And do you understand from that disclosure that Dr. Niyikiza was disclosing in his patent that the actual ranges of MMA lowering agent that are claimed are not critical to the invention?

MR. GROSSMAN: Objection to form of the question.

A. I don't -- I think the claims get more specific. So I would like to look at the actual claims.

BRUCE CHABNER

The method is to inject intramuscularly 1000 micrograms, and it says that the range is 500 to 1500 micrograms.

Q. Does the patent anywhere disclose why it is important to select those doses?

A. The patent sort of gives some flexibility to the physician, but I can tell you in practice everybody uses 1000.

Source: Ex. 1075 at 351:16-352:9; Paper 48 at 31.

Dr. Chabner's Testimony

Q. The patent discloses, "However, it will be understood that the amount of the MMA lowering agent actually administered will be determined by a physician in the light of the relevant circumstances,

including the condition to be treated, the chosen route of administration, the actual agent administered, the age, weight and response of the individual patient, and the severity of the patient's symptoms; and, therefore, the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases, still larger doses may be employed without causing any harmful side-effect."

Do you see that?

A. I do.

Q. And do you understand from that disclosure that Dr. Niyikiza was disclosing in his patent that the actual ranges of MMA lowering agent that are claimed are not critical to the invention?

MR. GROSSMAN: Objection to form of the question.

A. I don't -- I think the claims get more specific. So I would like to look at the actual claims.

Source: Ex. 1075 at 350:21-351:25; Paper 48 at 31.

Dr. Chabner's Testimony

BRUCE CHABNER

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Q. Does the patent anywhere disclose why it is important to select those doses?

A. The patent sort of gives some flexibility to the physician, but I can tell you in practice everybody uses 1000.

Source: Ex. 1075 at 352:1-9; Paper 48 at 31.

Dr. Chabner's Testimony

Q. Dr. Chabner, when did you work -- first hear that Eli Lilly was going to administer B12 and folic acid with pemetrexed?

A. B12 and folic acid? Well, I certainly knew, I heard about it when they got drug approval. I wasn't -- I don't think I was very cognizant of that trial that they were doing, the mesothelioma trial.

I probably knew about it. I'm not sure I knew that they had changed the regimen to the fully vitamin supplemented regimen during the trial, but I knew about it certainly when it was approved as a regimen by the FDA.

Q. And it was approved around February 20 -- 2004?

A. That's right, yes.

Q. And what were the circumstances of -- how did you learn about the combination of

the regimen?

A. I was called by The Wall Street Journal and asked, you know, what do you think of this regimen, and what do you think about the approval of the drug? And I said I was very surprised that this actually worked. I had a very short interview.

(Exhibit 2091 introduced.)

Q. I'm handing you a document that's been previously marked as Exhibit 2091, a Wall Street Journal article. Is this the article that you just referenced?

A. Yes.

Q. And if you turn to Page 3 of 5, the last sentence on that page. Or, not the last sentence.

A. Yeah. Last paragraph?

Q. The last paragraph. Last sentence of that paragraph.

You see where it is quoting you "When I first heard about it, I thought it was crazy, said Bruce H Chabner, clinical director of the Massachusetts General Cancer Center."

Dr. Chabner's Testimony

BRUCE CHABNER

A. Uh-huh.
Q. Do you see that?
A. Yes.
Q. And what was it that you thought was crazy?
A. That the idea that if you pretreated these patients with vitamin B12 and folic acid, that you would actually get -- you wouldn't reverse the antitumor activity.
Q. And the article, 2091, is dated April 21, 2004, right?
A. Right.
Q. When -- when did you speak to The Wall Street Journal reporter, shortly before that?
A. I think it was right at that time, yeah. It just came out very quickly afterwards.
Q. And so when you said that when I first heard about it, your recollection is you probably first heard about it a couple of months before April 20 --
A. You know, I -- I'm not really sure when I first heard about it. I mean I -- I

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was aware of the fact that they had tried folate supplementation. I can't even -- I can't recall when I first heard about B12 being added to a regimen and then this was added to the Phase III. I actually don't know.

I wasn't involved. I wasn't advising them about this. This was corporate business that wasn't public, as far as I was concerned.

Source: Ex. 1075 at 200:1-201:11; Paper 48 at 33.

Dr. Chabner's Testimony

Q. Prior to drug approval, did you ever have any conversations with anyone at Lilly about what they were doing with pemetrexed?

A. I don't recall. It's possible they might have mentioned to me the trial was going on and what they were doing, but I -- I certainly involved in it in any way.

Q. If someone from Lilly had mentioned it to you, would you have informed them of your skepticism?

A. Probably.

Q. And do you recall doing that?

A. But I, you know, I don't -- I don't know. I mean, you know, they had their own reasons for trying it, and I wasn't privy to the data. It wasn't in the -- the public domain at that time. I wasn't an advisor to Lilly. I never was one of their -- their important advisors on the pemetrexed stuff.

Q. When you first heard about the Lilly regimen that was approved --

A. Yeah.

Q. -- was it after 2001?

MR. GROSSMAN: Objection to form

of the question.

A. I just don't know exactly when I first heard of it.

I know that my opinions about folate pretreatment had been formed and were pretty solid. Up until the time it was approved I was very skeptical.

I had extensive experience with folate and reversal of methotrexate, and I just am -- I was aware of the lometrexol studies and I was aware of Hammond, I'm sure, and I was skeptical that it was going to work.

Q. You were aware of the lometrexol studies and you were aware of Hammond?

A. Yeah.

Q. How do you know you were aware of Hammond?

A. How do I know it? I don't know it. I'm not sure when I -- I think I was -- being a person -- I know I was aware of the lometrexol studies, but I -- I think that, you know, people -- we were keeping up with the literature and the abstracts so I'm sure

I did.

Dr. Chabner's Testimony

Q. And if you would flip to Page 370.
A. Yes.
Q. There's a heading -- there's a heading in that article that is "Safety and addition of folic acid in vitamin B12."
A. Yes.
Q. Do you see that?
Had you reviewed this article prior to the time you spoke to The Wall Street Journal?
MR. GROSSMAN: Objection to the form.
A. Had I reviewed it? I probably had read it.
Q. Did you have any of the disclosures from Exhibit 1047 in mind when you told The Wall Street Journal that you were very surprised?
A. From this -- this paper I don't think

changed my mind at all.

Dr. Chabner's Testimony

Q. And so if I understand you correctly, your views were formed about folate pretreatment based on your extensive work on methotrexate in the '70s and '80s?

A. That was certainly one of the major things. The other reason, I think, was the

Dr. Chabner's Testimony

A. I would point out that there is no clinical result in this paper that would change my mind. And I think you would have to agree.

You have not pointed out a clinical result with that regimen that was patented that would change your mind.

Q. What do you mean when you say there is no clinical result?

A. I mean there is no evidence that it worked in the clinical setting.

Q. And is evidence of something working in a clinical setting the criteria you employed to determine whether or not something would have a reasonable expectation of success?

MR. GROSSMAN: Objection to the form of the question.

A. Yes, because my -- my frame of mind was that it wasn't going to work, and this didn't prevent -- present any evidence to change that, and what was really needed was clinical evidence to change that mind -- my mindset about it.

Source: Ex. 1075 at 214:2-25; Paper 74 at 2.

Dr. Chabner's Testimony

A. Hammond I

96. As discussed previously, Hammond I would not provide the POSA with a reason to pre-treat with folic acid. To the contrary, Hammond I suggests that the use of folic acid pretreatment reduces the efficacy of pemetrexed, as when viewed in light of the earlier Hammond abstract (Hammond II) and Rinaldi I, the prior art indicated to the POSA that while 10 of 37 patients in the unsupplemented phase I study (Rinaldi I) showed a response, only 1 out of 33 patients in the folic acid pre-treatment phase I study (Hammond) showed a response. That comparison, and the resulting conclusion of a loss in efficacy, is supported by Laohavinij, which similarly observed a substantial reduction in the response rate despite a massive increase in lometrexol dosage between supplemented and unsupplemented phase I studies involving lometrexol. Laohavinij at 333. Hammond I would likewise discourage the POSA from using folic acid pre-treatment because the POSA would understand that the regimen resulted in an increase in kidney toxicity as compared to the Rusthoven study—an observation that the POSA would understand was based on the use of additional amounts of pemetrexed in order to compensate for the use of folic acid pre-treatment.

Source: Ex. 2120 ¶96; Paper 48 at 9.

Dr. Chabner's Testimony

157. Nonetheless, the data in Worzalla is consistent with the expectation of the POSA that folic acid pretreatment would be detrimental to the efficacy of pemetrexed, and would reinforce the POSA's understand that folic acid and vitamin B12 pre-treatment should not be utilized with pemetrexed. Specifically, Worzalla suggested that higher doses of pemetrexed would be required to compensate for the effects of folic acid, which for the reasons discussed previously would not be regarded as a viable clinical strategy by the POSA due to the expectation of kidney toxicity.

Source: Ex. 2120 ¶157; Paper 48 at 10.

Dr. Chabner's Testimony

164. First, although I understand that pemetrexed is technically covered by the generic structural formula in the '974 patent,[17] the POSA would not regard the disclosure of the '974 patent as being particularly relevant to pemetrexed. The '974 patent nowhere mentions pemetrexed or provides any data or example about it. Instead, it focuses on GARFT inhibitors such as lometrexol. While it has some GARFT inhibitory activity, pemetrexed is not a "GARFT inhibitor" as that term is used in the art. The POSA would understand the phrase "a GAR transformylase inhibitor" to refer to an antifolate whose primary locus of action is GARFT, such as lometrexol, LY309877, and AG2034. Pemetrexed's primary locus of action, however, is TS—not GARFT—and therefore would be regarded as a "TS inhibitor" by the POSA. *See, e.g.,* Jackman at 875-76 (noting that TS is likely the primary locus of action of pemetrexed and referring to pemetrexed as a "TS inhibitor"); Johnston at 11-12 (LY231514 listed as a TS inhibitor); Chu at 110 (same). And while pemetrexed was known as a "multi-targeted antifolate," and thus did have activity against GARFT, the POSA would not regard its GARFT activity to be particularly notable. *See, e.g.,* Shih at 1118 (pemetrexed was a potent inhibitor of rhTS and rhDHFR (although more potent for rhTS), but "only moderately inhibited rmGARFT", that is, was less potent against the enzyme); Graul at 502 (comparing K_i values for pemetrexed and its glutamate against TS, DHFR, and GARFT).

Source: Ex. 2120 ¶164; Paper 48 at 14, 27.

Dr. Chabner's Testimony

67. This concern still existed as of June 1999, and was not limited to leukemia. As one publication (Laohavinij) regarding pre-treatment with folic acid prior to administration of lometrexol to phase I patients with a variety of solid tumors observed, "One cause for concern is that the administration of folic acid prior to lometrexol and during treatment could potentially supplement the folate requirements of the tumour, and thereby circumvent the activity of lometrexol or, worse still, aid tumour progression." Laohavinij at 333 (citing Farber); *id.* at Table 1 (identifying the tumor types of the patients in the study).

Source: Ex. 2120 ¶67; Paper 48 at 17-18.

DR. ZEISEL

Dr. Zeisel's Testimony

23. Folic acid is the most common folate used as a dietary supplement, but it does not occur widely in nature. The human body converts it to so-called "reduced folates"—other forms of folate that participate in the folate cycle—using an enzyme called dihydrofolate reductase, or DHFR. Reduced folates also occur naturally in a variety of foods. *See* Combs at 379.

Source: Ex. 2118 at ¶23; Paper 48 at 12-13.

Dr. Zeisel's Testimony

Q Did you quantify efficacy in this Phase 1 trial with the prostate cancer patients?

A So like most Phase 1 trials, we are not powered to determined efficacy. But we did look at PSA levels of protein that is secreted

by many prostate tumor cells and rises in people with prostate cancer and asked did we lower it. And we had a number of responders -- I don't remember the exact number out of the group -- that lowered their PSA.

So that was a preliminary indication that it might be efficacy, and that's good that a Phase 1 trial can provide that. But proof of efficacy requires a study with adequate numbers of subjects to rule out random chance and null hypothesis.

So we did not draw conclusions about efficacy. Just said it appears that there may be efficacy.

Source: Ex. 1076 at 124:20-125:15; Paper 48 at 16.

Dr. Zeisel's Testimony

18	Q	What's the recommended daily intake
19	for folate?	
20	A	400 micrograms a day.

Dr. Zeisel's Testimony

Q But a POSA would have known that it was possible that nutritional status was the reason for being sensitive to pemetrexed toxicity; correct?

A A POSA would have known that the nutrients important for maintaining homocysteine concentrations at less than 10 micromolar would have been important in the nutrition of those people. I don't know about the other elements of nutritional status.

Source: Ex. 1076 at 40:10-19; Paper 48 at 19.

Dr. Zeisel's Testimony

that it is -- a POSA would have known that it's possible that low folate status, low B12 status, low betaine/choline status, low B6 status could have been contributing to higher homocysteines and that these people had and could have been but might not have been the reason for the high homocysteines they had and their risk for pemetrexed toxicity.

Source: Ex. 1076 at 41:5-12; Paper 48 at 19.

Dr. Zeisel

Dr. Zeisel's Testimony

Q What were the combination of nutrients that people with high homocysteine were treated with?

A Folate would have been a treatment that they used. Betaine would have been a treatment that they might have tried. B6, B12.

Q Anything else?

A Not that I recall.

Source: Ex. 1076 at 34:16-23; Paper 48 at 20.

Dr. Zeisel's Testimony

it wasn't, for the EP-005, they're addressing three of the four causes of elevated homocysteine that you mentioned earlier, which are low B6, low B12 and low folate; is that right?

A That's correct, yes.

Source: Ex. 1076 at 139:9-14; Paper 48 at 20.

Dr. Zeisel's Testimony

piece, I have said that there are numbers of reasons that homocysteine could be elevated, B12 or low B12 being one of them.

However, in the presence of normal MMA, I believe that Niyakiza does not support the conclusion that B12 is -- B12 deficiency is the cause. It just says that, at least in this group of patients, I can't say it is and I can't rule out it isn't.

Source: Ex. 1076 at 116:12-20; Paper 48 at 24.

Dr. Zeisel's Testimony

that it is -- a POSA would have known that it's possible that low folate status, low B12 status, low betaine/choline status, low B6 status could have been contributing to higher homocysteines and that these people had and could have been but might not have been the reason for the high homocysteines they had and their risk for pemetrexed toxicity.

Source: Ex. 1076 at 41:5-12; Paper 48 at 25.

Dr. Zeisel's Testimony

Q But for patients where it makes sense, you would give -- if you are going to treat the patient with folate, you would also treat the patient with B12 to account for potential masking?

MR. KRINSKY: Object to the form; asked and answered.

THE WITNESS: Yes, for a -- the clinical judgment, I would give folate and B12, because I would have made a judgment in that specific patient situation that the folate and B12 weren't going to have a -- counteract another intervention that I was going to be giving.

Source: Ex. 1076 at 98:4-17; Paper 48 at 25-26.

Dr. Zeisel's Testimony

> And so, again, people -- I don't recall of a clinical study in which they did a randomized control trial with tumor growth or a clinical study in which they gave B12 and asked whether B12 or no B12, there was a difference in tumor growth rate.

Source: Ex. 1076 at 145:17-22; Paper 48 at 27.

Dr. Zeisel's Testimony

Q And when you cited portions of Vidal, which you say is similar to the PDR, did you look at the PDR to see if it contained equivalent disclosures?

STEVEN H. ZEISEL, M.D., Ph.D.

A I did.

Q And what did you find?

A The PDR didn't state that B12 was contraindicated because of its effect on rapidly dividing cells.

Q I'm sorry. I didn't catch the last part.

A What I said is that Vidal says that B12, vitamin B12 is contraindicated in patients because B12 can give rise to exacerbation of cancer progress, and the PDR did not include that line.

Q Did the PDR say anything about vitamin B12 being contraindicated in cancer patients?

A No.

Source: Ex. 1076 at 41:22-42:17; Paper 48 at 29.

Dr. Zeisel's Testimony

18	Q	What's the recommended daily intake
19	for folate?	
20	A	400 micrograms a day.

Dr. Zeisel's Testimony

Q What's the reason for using an intramuscular dose?

A A patient who can't absorb an oral dose would get an intramuscular dose.

Q Is it common for B12 patients not to be able to absorb an oral dose?

A For patients with pernicious anemia, this subset of patients we've just been talking about, their problem is is they do not make a protein in the gut needed to intramuscular B12.

So they cannot absorb B12. And you treat people with that problem with intramuscular dose.

Q Are you aware of what the standard intramuscular B12 dose is?

MR. KRINSKY: Object to the form; foundation, scope.

THE WITNESS: In this -- I don't know what the standard was in 1999. In

this paper, in 1998 -- in 1988, they are using -- let's see if I can find it in here.

I'm not sure I can find what dose they used in this amount at this time. So I can't tell you what -- it's somewhere in here but I haven't had time to note it. Perhaps you know where they tell you the dose in this paper.

BY MS. SPIRES:

Q I'm less concerned with --

A Okay.

Q -- this particular paper and more concerned with, as a practicing nutritionist, what you believe to be the standard intramuscular dose for vitamin B12.

A It varies. And it would have been -- you need about 4 micrograms. But you would have given a dose that is milligrams, probably at that time, as an intramuscular dose.

So that would be 10 to -- you know, or more times what the normal oral dose would have been, because it's to serve as a depot

Dr. Zeisel's Testimony

STEVEN H. ZEISEL, M.D., Ph.D.

that they can absorb from for a while.

And so it doesn't really matter how much you give intramuscularly; it's just how long it lasts before they lose it by not being able to reabsorb the amount that they excrete into their gut.

Q What do you mean when you say "it doesn't really matter how much you give intramuscularly"?

A So you only need a few micrograms. That's available from the intramuscular dose.

The problem is, for people who can't absorb it every day, they are secreting B12 into their intestine, and then they can't reabsorb it. So they rapidly run themselves down.

And so when you give an IM dose, you don't have to give it to them daily. You give them something more than the few micrograms they need so that they can draw on that dose that's sitting in the muscle that you stuck it in for a period of time.

And so a standard dose probably, you know, a milligram would have been enough to

STEVEN H. ZEISEL, M.D., Ph.D.

last them weeks before they run it down. A microgram being a millionth of a gram, and a milligram being a thousandth of a gram.

Q I'm sorry. I didn't catch the last part.

A A milligram is a thousandth of a gram; and a microgram is a millionth of a gram. So a milligram is a lot of micrograms.

Q And that dosing was true in 1999; correct?

Source: Ex. 1076 at 90:1-91:11; Paper 48 at 31.