MEDICAL PROGRESS

THE PHARMACOLOGY AND CLINICAL USE OF METHOTREXATE

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METHOTREXATE, the most widely used antimetabolite in cancer chemotherapy, has an essential role in the treatment of such diverse diseases as acute lymphocytic leukemia, non-Hodgkin's lymphoma, osteosarcoma, choriocarcinoma, head and neck cancer, and breast cancer.[1] It has also become an important therapeutic alternative in the treatment of severe psoriasis[2] and in the suppression of graft-versus-host disease after bone-marrow transplantation,[3] as well as in the experimental treatment of various rheumatic diseases after primary therapy has failed.[4] Through pharmacologic research we have gained an understanding of the basic steps in the action of methotrexate, including its transport across cell membranes, its intracellular conversion to a series of polyglutamate metabolites, its potential multiple sites of action, and of greatest interest, the biochemical and genetic changes that lead to resistance to the drug. This pharmacologic knowledge has led in turn to clinical experimentation with new sequences and combinations of methotrexate and with high-dose regimens, in an effort to overcome resistance. In this article we review the important new concepts of methotrexate action and describe their impact on the clinical use of the drug, with particular emphasis on the rationale and results of high-dose methotrexate regimens.

BIOCHEMICAL PHARMACOLOGY

The folate vitamins are a class of essential cofactors that carry one-carbon groups. These one-carbon elements are required for the synthesis of both purines and thymidylic acid, which in turn are essential for DNA synthesis and cell division. Thus, in the design of inhibitors of DNA synthesis, folic acid and its derivatives are a logical target.

The physiologic folate cofactors all share the common structural features shown in Figure 1. This structure consists of three elements: a multi-ring pteridine group linked to para-aminobenzoic acid, which in turn connects with a terminal glutamic acid residue. Although folates found in the blood have a single terminal glutamate, most intracellular folates are converted to polyglutamates, which contain multiple glutamate groups linked by gamma-peptide bonds.[5,6] The polyglutamate forms of folic acid have unique properties. They are preferentially retained inside the cells and are usually more efficient cofactors than the monoglutamated compounds.[6] A second important feature of folate biochemistry is that the cofactors must be reduced to their tetrahydro form, with hydrogens in positions 5, 6, 7, and 8 of the pteridine ring (tetrahydrofolates [FH_4]), in order to be active in enzymatic reactions. (Fig. 1A). The enzyme dihydrofolate reductase, which is responsible for conversion of oxidized folates to their active, reduced form, is potently inhibited by methotrexate.[7]

Since their clinical introduction in 1948, antifolates have become widely used as chemotherapeutic agents.[8] Methotrexate, the 4-NH_2, N-10 methyl analogue of folic acid (Fig. 1), has been the most widely used antifolate. Current concepts of the drug's mechanism of action are illustrated in Figure 2. Methotrexate enters cells through the active transport system used by the physiologic circulating folate N^5-methyl-FH_4 and N^5-formyl-FH_4 (leucovorin and folinic acid), which is used as a rescue agent after high-dose therapy.[9-11] In addition to active transport, a second drug entry mechanism comes into play at high concentrations of methotrexate (in excess of 20 μM).[12,13] This poorly characterized process, which probably involves diffusion, is less efficient than the active transport process, but it accounts for the major fraction of drug that enters cells at high concentrations and explains the ability of "transport-resistant" cells to take up methotrexate at high extracellular concentrations. This carrier-independent uptake provides a rationale for the clinical use of high-dose methotrexate. As an alternative approach to circumvention of transport resistance, lipid-soluble antifolates, which readily diffuse across the cell membrane,[14,15] are being developed for clinical use. After entering the cells, methotrexate quickly binds to and inactivates dihydrofolate reductase. This enzyme has a crucial role in maintaining intracellular FH_4 pools by reducing dihydrofolic acid (FH_2), which is produced during thymidylate synthesis. Since this is the only reaction that converts the reduced folate to the inactive oxidized FH_2, the underlying rate of thymidylate synthesis is an important determinant of cytotoxicity.[16-19]

An excess of unbound, or "free," methotrexate, above the amount required for simple titration of dihydrofolate reductase binding sites, is required to block FH_2 reduction. In the presence of active thymidylate synthesis, the inhibition of dihydrofolate reductase leads to an accumulation of its substrate FH_2. High FH_2 concentrations then compete with methotrexate for binding to dihydrofolate reductase and tend to diminish the drug's effectiveness[20-22] unless excess free methotrexate is present.

The critical result produced by inhibition of dihydrofolate reductase is depletion of intracellular pools of reduced folate. The reaction most sensitive

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to folate depletion is thymidylate synthesis, which requires $N^{5\text{-}10}$methylene-FH_4. This reaction ceases at concentrations of 1×10^{-8} M of methotrexate.[23] N^{10}-formyl-FH_4, the folate involved in both folate-dependent steps of purine synthesis,[24] is also depleted, leading to the cessation of purine synthesis at slightly higher concentrations (approximately 1×10^{-7} M) of methotrexate.[25] The lack of either thymidylate or purines blocks synthesis of DNA.

Like the physiologic folates, methotrexate is extensively metabolized intracellularly to polyglutamate derivatives (Fig. 1 and 2). Baugh et al.[26] first observed these derivatives in red cells, and other investigators subsequently found them in the livers of patients who had received methotrexate.[27] The compounds have now been identified in various murine and human tissues.[27-40] Methotrexate polyglutamate synthesis increases with increases in drug concentration and duration of exposure. In human breast-cancer cells, notable formation of methotrexate polyglutamate occurs only after six hours of incubation at a concentration of 2 μM methotrexate — a concentration–time profile easily achieved with high-dose methotrexate therapy but not with small "conventional" doses[40,41] of 15 to 50 mg per square meter of body-surface area. Higher concentrations of drug given for longer periods of time lead to progressive lengthening of the polyglutamate chain and larger amounts of methotrexate polyglutamate formation in comparison with levels of the parent drug.

The formation of methotrexate polyglutamates is important for several reasons. It allows accumulation of free intracellular drug far above the levels of the parent compound that would otherwise exist in equilibrium with extracellular drug. These compounds have at least equal affinity for dihydrofolate reductase,[42,43] but in intact cells the methotrexate polyglutamates appear to dissociate from dihydrofolate reductase at a slower rate than methotrexate, indicating that they are potentially less reversible inhibitors than

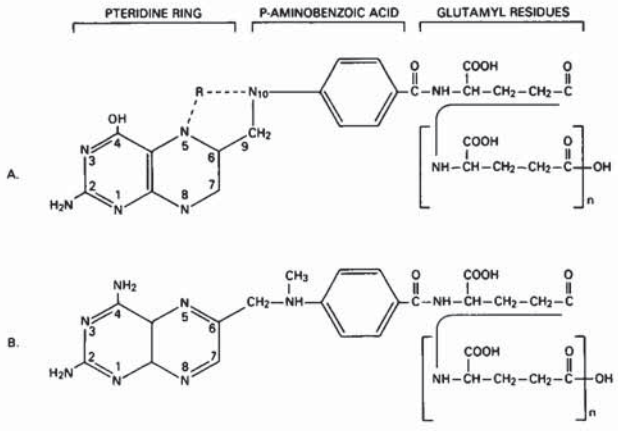

Figure 1. Structure of Tetrahydrofolate (A) and Methotrexate (B) Polyglutamates.
In Panel A one-carbon groups (R) are transported on nitrogen 5 or 10 or both.

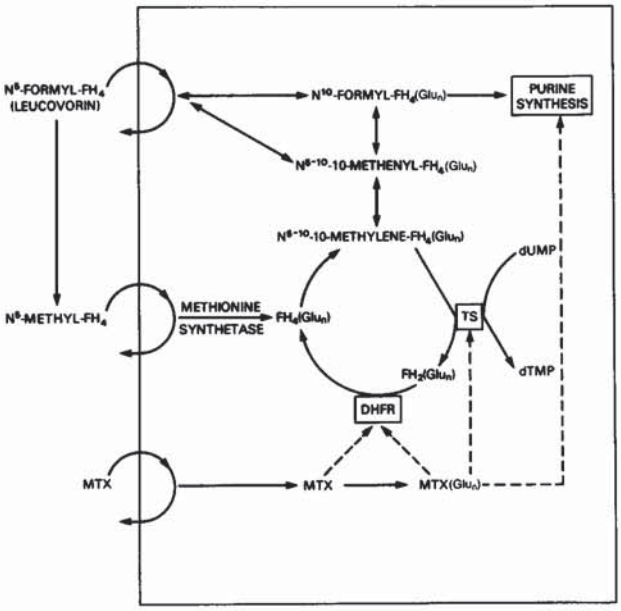

Figure 2. Mechanism of Action of Methotrexate.
MTX denotes methotrexate, DHFR dihydrofolate reductase, TS thymidylate synthetase, FH_4 tetrahydrofolate, FH_2 dihydrofolate, Glu glutamyl, dTMP thymidylate, and dUMP dioxyuridylate. Broken lines indicate enzyme inhibition.

the parent drug. Further studies of the interaction of methotrexate polyglutamates with purified enzyme will be required to clarify differences between the parent compound and metabolites.

The most striking property of the polyglutamates is their ability to remain within the cell in the absence of extracellular drug,[36,40,41,44,45] in contrast to the parent compound, which rapidly leaves the cells after the extracellular drug disappears. Retention is clearly influenced by chain length: the derivatives that contain three or four additional glutamates are retained for up to 24 hours in the absence of external drug,[41] whereas the compounds with a shorter chain length have proportionately shorter retention times. Longer retention is associated with prolonged inhibition of dihydrofolate reductase and extended cytotoxicity.[40]

In addition to inhibiting dihydrofolate reductase, the methotrexate polyglutamates inhibit other folate-requiring enzymes not affected directly by methotrexate. It is well established that physiologic folate polyglutamates have a much greater affinity for folate-requiring enzymes than do the corresponding monoglutamated derivatives.[5] Preliminary experiments have shown that the addition of one glutamyl residue to methotrexate transforms the drug into a potent direct inhibitor of both thymidylate synthetase[46] and aminoimidazolecarboxamide ribonucleotide transformylase[47]; the latter is one of the enzymes involved in de novo purine synthesis.

Resistance to Methotrexate

On the basis of the understanding of methotrexate action outlined above, it is possible to compare the

biochemistry of sensitive and resistant tumor cells, with the hope of defining the determinants of response and devising treatment strategies to overcome resistance. From studies of experimental methotrexate resistance, it is clear that resistant cells may display any one or more of the following changes: decreased membrane transport of methotrexate, altered dihydrofolate reductase, which has reduced affinity for methotrexate, increased levels of dihydrofolate reductase due to amplification of the gene controlling dihydrofolate reductase synthesis, decreased formation of methotrexate polyglutamates, and decreased activity of thymidylate synthetase.

Decreased Membrane Transport

Tumor cells exposed to methotrexate in vitro may become drug-resistant because of impairment of the active uptake system[13,48] through a decrease in the affinity of the carrier for methotrexate. This type of resistance has not been clearly documented in patients with resistance to methotrexate.

Decreased Affinity of Dihydrofolate Reductase for Methotrexate

Experimental tumor cells resistant to methotrexate may contain dihydrofolate reductase with decreased binding affinity for methotrexate — a mechanism demonstrated in various mouse[49-53] and human[54] cell lines. In a group of five mouse leukemias not previously exposed to methotrexate, Jackson and Niethammer[54] found a correlation between cytotoxicity and dihydrofolate reductase affinity for methotrexate — i.e., the lower the affinity for methotrexate, the less the cytotoxicity. The altered dihydrofolate reductase may differ in molecular weight[52] from wild-type enzyme, may have an affinity for methotrexate that is decreased from 2.5- to 270-fold,[50,51,54] and may retain its affinity for antifolates other than methotrexate.[55] Whether altered dihydrofolate reductase causes drug resistance in human tumors is still uncertain.

Increased Levels of Dihydrofolate Reductase

One of the most interesting ways in which cells become resistant to antitumor agents is through the process of gene amplification. It is now well established that genes coding for target proteins such as dihydrofolate reductase may amplify by unknown mechanisms, leading to overproduction of the target enzyme and drug resistance. Since the early studies of Harding et al.,[56] it has been known that dihydrofolate reductase levels in both normal hematopoietic cells and leukemic cells can increase during the course of treatment with methotrexate. Striking increases in dihydrofolate reductase levels can be produced in tumor cells in culture by long-term exposure to stepwise increasing concentrations of methotrexate.[56-64] It is important to note that overproduction of dihydrofolate reductase may occur as an isolated resistance mechanism or in conjunction with other changes, such as decreased transport[48,65,66] or altered dihydrofolate reductase with a decreased affinity for methotrexate.[51,67]

The initial evidence of a genetic basis for increased dihydrofolate reductase was reported by Biedler and Spengler, who observed expanded homogeneously staining regions in elongated chromosomes from methotrexate-resistant cells.[68] Other workers subsequently discovered that such cells contain increased copies of mRNA and, finally, increased numbers of genes coding for dihydrofolate reductase.[52,69-76] These amplified dihydrofolate reductase genes are located either in homogeneously staining regions (Fig. 3A) or in small bodies of extrachromosomal DNA, termed "double minutes"[77,78] (Fig. 3B). Both changes can be visualized by standard karyotyping of resistant cells, and double minutes have been reported in human tumor-cell lines isolated from patients who have received methotrexate.[78]

The type of cytogenetic abnormality present in drug-resistant cells determines the stability of resistance. Since double minutes lack centromeres, they segregate unequally into daughter cells during mitosis. In the absence of selective pressure, such as that from the cytotoxic drug, the daughter cells containing double minutes do not have a growth advantage and lose their dominant place in the cell population. In contrast, chromosomes containing homogeneously staining regions appear to duplicate normally, and resistance mediated by the regions is stable in the absence of drug. This finding suggests that tumors resistant to methotrexate on the basis of double-minute–mediated gene amplification may lose their additional dihydrofolate reductase genes and revert to sensitivity if the drug is withdrawn. This sequence of reversion of double-minute–mediated resistance to sensitivity during extended periods in tissue culture has been observed in a tumor-cell line isolated from a patient with small-cell carcinoma who was treated with methotrexate.[78]

Decreased Polyglutamation

Although it is clear that polyglutamation allows enhanced accumulation of free drug, extends drug action, and promotes inhibition of additional enzymes, evidence that the absence of polyglutamation may be responsible for resistance is still preliminary. A resistant human breast-cancer cell line in which decreased polyglutamate formation played a part in drug resistance has been described elsewhere.[78a] The biochemical defect in this resistant cell line has not been extensively characterized.

Thymidylate Synthetase Activity

As noted above, many investigators have found that the underlying rate of thymidylate synthesis is an important determinant of methotrexate cytotoxicity. Lower rates of thymidylate synthesis make cells less susceptible to methotrexate.[16-19] Further studies of

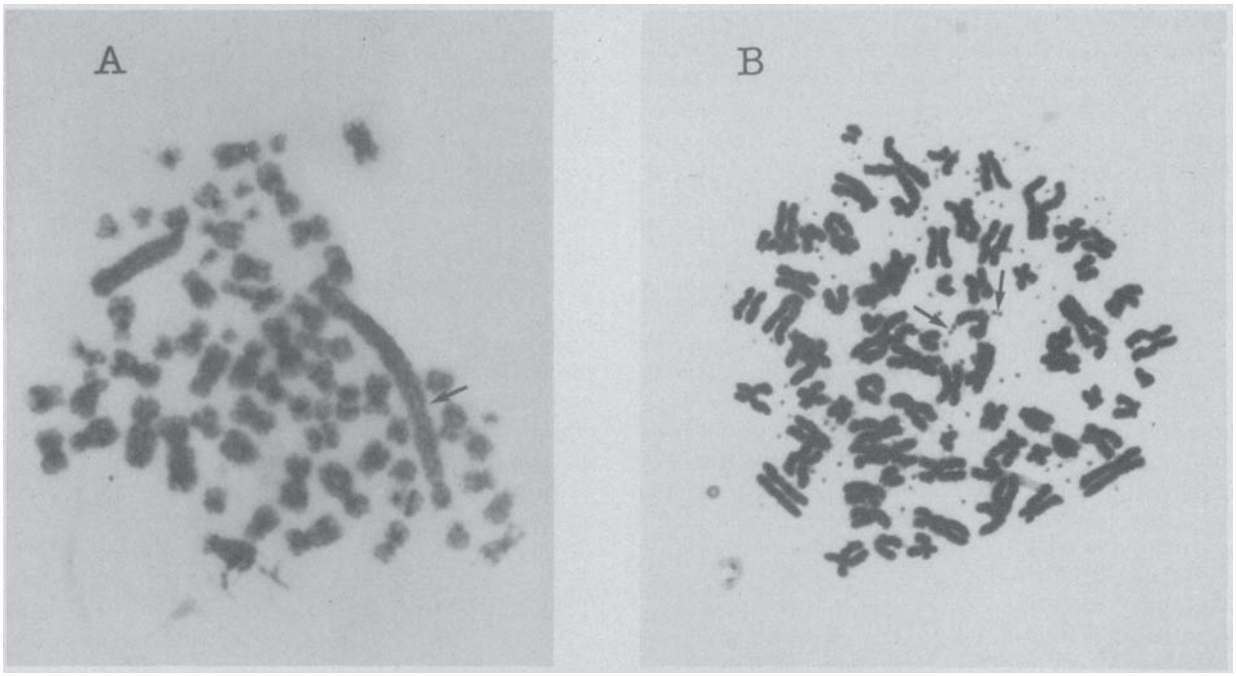

Figure 3. Metaphase Plates of Methotrexate-Resistant MCF-7 Human Breast Cancer Cells (A) and NCI-H249P Human Lung Oat-Cell Carcinoma Cell Line (B).

Arrow in Panel A indicates a marker chromosome with a greatly expanded homogeneously staining region. In Panel B arrows show prominent double-minute chromosomes.[78]

human tumors will be required to determine the role of this pathway in clinical methotrexate resistance.

Clinical Pharmacology

Although the primary features of methotrexate pharmacokinetics in animals have been known for 10 years,[79] only in the past few years has this knowledge been systematically applied to the design and monitoring of clinical treatment regimens. Two critical elements were necessary for this application: an understanding of the relation between pharmacokinetic variables and drug effects, and the development of rapid, sensitive, and specific assays that allow routine drug monitoring. The threshold for the sensitivity of human tumor cells to methotrexate has not been established, but it is clear that drug concentrations much higher than 10^{-8} M may be required to inhibit DNA synthesis in resistant cells, and that cells with greatly increased dihydrofolate reductase levels may be resistant to drug concentrations above achievable plasma levels (>1 mM).[64,69]

In vitro experiments have established that above the threshold concentration required for cytotoxicity, cell kill increases with increasing drug concentrations.[80] This may be due to the production of polyglutamate metabolites of methotrexate or may result from increased diffusional uptake or more complete saturation of dihydrofolate reductase. Thus, both empirical and theoretical considerations concerning cytotoxicity and drug resistance support the employment of regimens that achieve drug concentrations above 1×10^{-6} M.

In addition to the important role of drug concentration, a second pharmacokinetic variable — duration of tumor-cell exposure — is a critical determinant of cytotoxicity. In general, cytotoxicity is directly proportional to duration of exposure. Brief periods of exposure (under six hours) are less effective than longer periods in producing cell kill.[80,81] Thus, the commonly used human tumor stem-cell assay has potentially limited value for defining clinically useful thresholds of methotrexate sensitivity, because the conditions chosen (exposure for one hour in medium containing thymidine and purine sources) tend to minimize cell kill.

The second necessary element in a rational treatment design is a method for monitoring drug concentrations in plasma.[82-86] Newer methods, including competitive protein binding,[83] radioimmunoassay,[84] and the enzyme-linked immunoassay,[85] have made it possible to compose a detailed picture of methotrexate pharmacokinetics in the clinical situations described below.

Oral Maintenance Therapy in Acute Lymphocytic Leukemia

Numerous pharmacokinetic studies have shown that both the rate and the total amount of drug absorption after oral administration are quite variable among patients.[87-90] With optimal absorption, doses of 20 mg per square meter produce peak blood levels of

approximately 1 μM. The peak occurs between one and five hours after drug administration, and drug levels remain at above 1×10^{-7} M for approximately six hours.[87] With the doses used for maintenance chemotherapy in acute leukemia (15 to 25 mg per square meter, once weekly), impairment of drug absorption can produce an ineffective drug-concentration profile in plasma, with peak drug levels below 5×10^{-7} M.

There is preliminary evidence that among patients with acute lymphocytic leukemia who have a good prognosis, clinical relapse occurs more frequently in those who have delayed absorption of oral methotrexate. In one study, relapse occurred in 14 per cent of patients with "fast" absorption but in 32 per cent of those with "slow" absorption.[87] This possible explanation for leukemic relapse requires further study and more sophisticated pharmacokinetic evaluation to document the proposed relation between absorption and relapse. Such studies are in progress.

At higher doses, there is strong evidence for reduced methotrexate bioavailability. For example, the bioavailability of 50 mg per square meter is only 20 to 50 per cent, as determined by a pharmacokinetic comparison of plasma drug concentration after oral versus intravenous administration.[91] With still larger doses, absorption decreases to approximately 25 per cent at 200 mg per square meter.

Distribution of Methotrexate in the Central Nervous System

The distribution of methotrexate has important implications for its clinical use. As a weak organic acid, methotrexate is negatively charged at neutral pH, has limited lipid solubility, and therefore diffuses slowly across physiologic membranes. This property limits the drug's ability to cross into the cerebrospinal fluid[92] or into third-space fluid collections, such as pleural effusion and ascites.[93] At steady state during a constant intravenous infusion, drug levels in the cerebrospinal fluid are 3 per cent of those in plasma, leading to a steady-state gradient of 30:1 between plasma and cerebrospinal fluid. Methotrexate exits from the cerebrospinal fluid by two mechanisms: as a passive passenger in the reabsorption of cerebrospinal fluid (bulk flow), and by an active transport process that can be inhibited by raised intracranial pressure or by probenecid.[94] It is common practice to administer methotrexate directly into the cerebrospinal fluid, since penetration from the systemic circulation is so limited. However, intrathecal methotrexate in combination with cranial irradiation is associated with chronic neurotoxicity (cortical thinning, intracerebral calcification, and mild dementia).[95] Alternative regimens employing systemic methotrexate without irradiation are now being studied for central-nervous-system prophylaxis in acute lymphocytic leukemia. Intravenous infusions of extremely large doses (15 to 30 g per square meter) of methotrexate are required to achieve cerebrospinal-fluid drug levels that approximate those achieved by intrathecal therapy with 12 mg. Regimens employing lower doses of methotrexate (500 mg per square meter) have not been effective in preventing meningeal leukemic relapse,[96] probably in part because of substantial interpatient variability in the level achieved in the cerebrospinal fluid.[97]

High-dose systemic administration has the additional advantage of providing a more uniform distribution of drug to the brain and ventricular fluid. Drug injected into the lumbar intrathecal space is distributed poorly to the ventricles, resulting in concentration gradients of 1000:1 or higher between the lumbar and ventricular fluid.[92] Distribution is further impaired if the patient is allowed to assume an upright position immediately after the lumbar puncture.[98] The relative efficacy and toxicity of systemic versus intrathecal methotrexate regimens are now being evaluated in ongoing trials with patients who have childhood leukemia.

Clinical Use

Antineoplastic Activity

Methotrexate has antitumor activity against a wide variety of tumors (Table 1). However, the drug is used most often in combination chemotherapy regimens. Examples of combinations based on an experimental rationale are listed in Table 2.

High-Dose Methotrexate Regimens with Leucovorin Rescue

In the past decade, methotrexate has been used more frequently in high doses (1 to 30 g per square meter) followed by leucovorin (N^5-formyl-FH_4, folinic acid) rescue in the hope of increasing the drug's therapeutic index. Leucovorin probably rescues cells by repleting intracellular FH_4 pools. It is rapidly metabolized in plasma to N^5-methyl-FH_4, the physiologic circulating folate.[107] To enter the FH_4 pools, N^5-methyl-FH_4 must first donate its methyl group by acting as a cofactor in methionine synthesis (Fig. 2).[108,109] Consequently, the effectiveness of leucovorin rescue will depend on the levels of methionine synthetase in the target tissues.[109]

Rationale

The use of high doses of methotrexate leading to high plasma levels (10^{-4} to 10^{-5} M) for prolonged periods (12 to 36 hours) has several appealing features based on the drug's biochemical pharmacology: (1) at high plasma levels, passive entry of methotrexate into tumor cells can potentially overcome drug resistance due to defective active transport; (2) the increased free intracellular methotrexate levels achieved can overcome drug resistance secondary to increased dihydrofolate reductase or altered enzyme binding; (3) the high and prolonged plasma drug levels can promote increased methotrexate polyglutamate formation, resulting in more prolonged drug action and accessory sites of action; and (4) prolonged drug administration can expose more cells to methotrexate during DNA synthesis. Furthermore, high-dose therapy may prevent or delay methotrexate resistance,[110] since in ex-

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