ARTHRITIS & RHEUMATISM
Vol. 50, No. 5, May 2004, pp 1370–1382
DOI 10.1002/art.20278

REVIEW

Toward a Better Understanding of Methotrexate

Joel M. Kremer

More is known about the metabolism, toxicity, pharmacokinetics, and clinical profile of methotrexate (MTX) than any other drug currently in use in either rheumatology or oncology. In the 56 years since Farber et al first described clinical remissions in children with acute leukemia after treatment with the folate antagonist aminopterin (1), antifolate drugs, dominated by MTX, have been used to treat millions of patients with malignant and autoimmune diseases. It is estimated that MTX is now prescribed to at least 500,000 patients with rheumatoid arthritis (RA) worldwide, making it by far the most commonly used disease-modifying antirheumatic drug (DMARD). Indeed, MTX is prescribed for more patients with RA than are all of the biologic drugs in current use combined. It is the most commonly studied and prescribed agent used in combination with other DMARDs, where clear additive therapeutic value is demonstrated (2–8).

In spite of our collective experience and success with MTX in the last 20+ years, the overall level of sophistication regarding the many issues and complexities associated with its use is surprisingly thin. Dogma about maximum weekly dosages, use in the elderly, monitoring with blood tests, and when to "give up" and add other agents to an MTX regimen is often invoked without rigorous scientific support. A sound understanding of the drug's many cellular effects is sometimes viewed as irrelevant to its use for rheumatic disease.

These behaviors and philosophies are understandable, since very busy clinicians often do not have the time or resources to research every nuanced publication describing the best way to conceptualize clinical decisions regarding the use of an agent such as MTX, which is supported by an enormous and often complex literature. It is the goal of this contribution to review the major and significant concepts regarding MTX metabolism which may be relevant to the treatment of rheumatic disease, not to summarize publications about its clinical effects. It will become apparent in the course of the discussion that most of what we know about the metabolism of MTX is derived from the oncology literature. Clinicians who have become familiar with the concepts presented should be better equipped to prescribe the drug in a more effective and rational manner. In addition, emerging insights into the role of naturally occurring genetic variations in cellular pathways of MTX metabolism hold promise for predicting both efficacy and toxicity of the drug. In spite of the real and perceived gaps in our understanding of the effects of MTX, at the time of this writing it remains a cornerstone for the treatment of RA and other rheumatic conditions, now and for the foreseeable future.

Joel M. Kremer, MD: Albany Medical College, and The Center for Rheumatology, Albany, New York.

Address correspondence and reprint requests to Joel M. Kremer, MD, The Center for Rheumatology, 1367 Washington Avenue, Albany, NY. E-mail: jkremer@joint-docs.com.

Submitted for publication October 1, 2003; accepted in revised form February 12, 2004.

Cellular effects

As described by Chu and Allegra in their excellent review of the metabolic actions of MTX (9), there are only 3 specific tetrahydrofolates that play essential roles as 1-carbon carriers involved in the synthesis of DNA precursors. The first, 10-formyltetrahydrofolate (10-formyl-THF), provides its 1-carbon group for the synthesis of purines in reactions mediated by glycinamide ribonucleotide (GAR) transformylase and aminoimidazolecarboxamide ribonucleotide (AICAR) transformylase (Figure 1). A second cofactor, 5,10-methylenetetrahydrofolate (CH_2-THF), donates its 1-carbon group to the reductive methylation reaction converting dUMP to thymidylate dTMP (Figure 2). A third, 5-methyltetrahydrofolate (5-CH_3-THF), donates a methyl group in the conversion of homocysteine to methionine and will be discussed below. These effects of MTX on purine metabolism and the de novo synthesis of DNA via inhibition of thymidylate production are essential to the understanding of the effects of MTX.

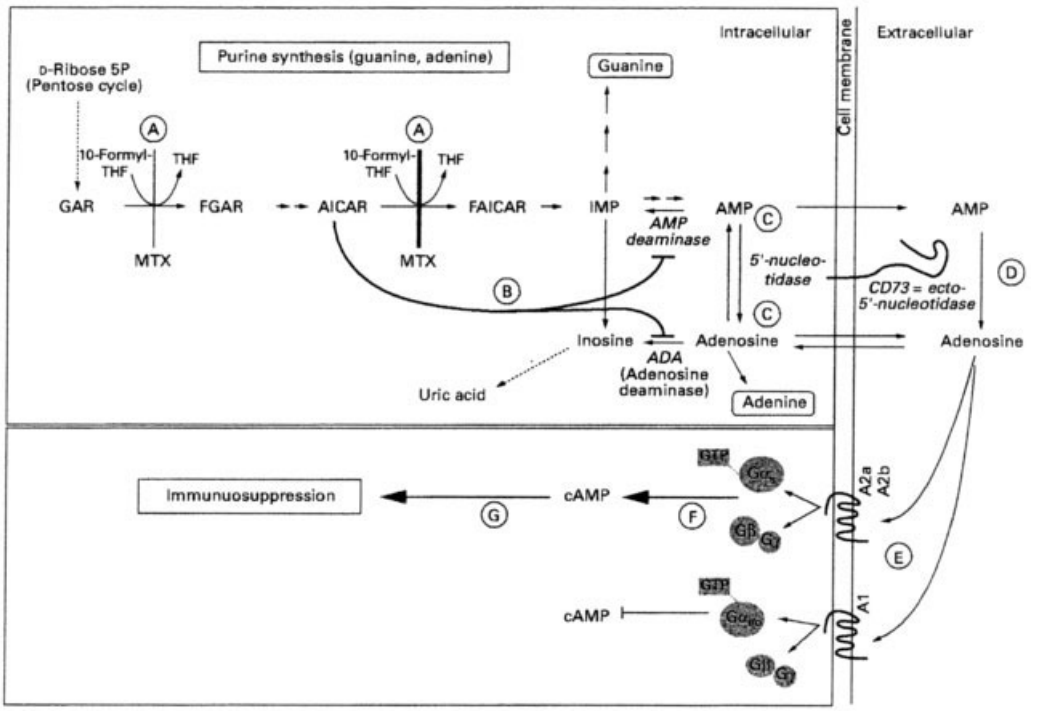

Figure 1. Effects of methotrexate (MTX) on purine metabolic pathways (**top**), and binding of adenosine to receptors and subsequent effects (**bottom**). **A,** MTX inhibits both conversion of glycinamide ribonucleotide (GAR) to 10-formyl GAR (FGAR) and conversion of aminoimidazole carboxamide ribonucleotide (AICAR) to 10-formyl AICAR (FAICAR). However, inhibition of the second step is stronger, which results in accumulation of AICAR. **B** and **C,** Accumulated AICAR inhibits AMP deaminase and ADA, which increases adenosine-5′-P and adenosine. **D,** Intracellular accumulation of adenosine-5′-P and adenosine results in an increase of these compounds in the extracellular space, where **E,** adenosine-5′-P is converted to adenosine, which binds to the specific receptor subtypes A1, A2a, and A2b. **F,** Probably, there will be a preponderance of the A2 receptor pathway, yielding an increase of cAMP in the cell. **G,** Increased cAMP leads to immunosuppression. 10-formyl-THF = 10-formyltetrahydrofolate. Reproduced, with permission, from ref. 95.

Besides yielding a 1-carbon group, CH_2-THF is oxidized to dihydrofolate (DHF), which can in turn be reduced back to THF by dihydrofolate reductase (DHFR) (Figure 2).

A variety of the effects of MTX on other pathways have potential relevance as we attempt to associate specific cellular events with resultant efficacy and toxicity.

Transmembrane transport

It has been previously accepted that MTX enters cells solely via the reduced folate carrier (RFC), which has a ubiquitous distribution (10) (Figure 3). MTX and leucovorin (5-CHO-THF) compete for uptake using the same active transport mechanism, a process of anion exchange involving the RFC. Members of another membrane transport group of proteins, termed folate receptors (FRs), are variably expressed and typically are responsible for the transport of folic acid and 5-CH_3-THF, the form of folate found in foods (11). RFCs and FRs can be expressed either separately or simultaneously in the same cell.

The membrane fluid bilayer comprises specific lipid associations involving sphingolipids and cholesterol, termed "rafts," that mediate interorganelle transport of membrane proteins (12). FRs exist within these lipid rafts as a family of 3 glycosyl-phosphatidylinositol–anchored glycoproteins. Interestingly, normal tissues express low or undetectable levels of the 3 FRs (13). However, FRs may be up-regulated in cells with increased metabolic activity, including malignant tissue (14) and synovial macrophages (15,16).

Synovial tissue from patients with RA express FRs (15). In this setting, FRs may also serve as a significant conduit for MTX influx. Scintigraphy of FRs has actually been reported as a useful imaging modality for identification of activated synovial macrophages

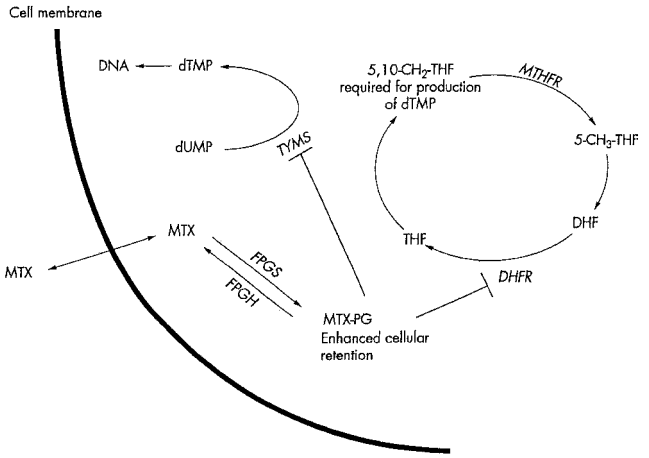

Figure 2. Methotrexate (MTX) polyglutamation affects thymidine nucleotide production and inhibits dihydrofolate reductase (DHFR). Effects of 5,10-methylenetetrahydrofolate (5,10-CH_2-THF) as a cofactor for the regeneration of DHF via the intermediary 5-methyltetrahydrofolate (5-CH_3-THF) (also found in food) are also depicted. MTX is retained within the cell in its polyglutamated form (MTX-PG). The enzyme folylpolyglutamate synthetase (FPGS) adds glutamic acid moieties, while folylpolyglutamate hydrolase (FPGH) removes them. MTHFR = methylene tetrahydrofolate reductase; TYMS = thymidylate synthase. See text for details. Reproduced, with permission, from ref. 27.

(16). There thus appears to be some blurring of the previously accepted clear delineation of the function of RFCs and FRs, with the latter capable of transporting MTX in cells in the physiologically and metabolically up-regulated state found in the rheumatoid synovium. As has been suggested (16), this phenomenon of up-regulation of the expression of FRs on synovial macrophages could be used to design a more selective folate antagonist utilizing only FRs for intracellular transport, with the resultant possibility of decreased systemic toxicity.

Interestingly, the membrane folate-binding protein has been shown to be markedly up-regulated by folate depletion and conversely, down-regulated in folate-replete medium (17). In conditions of high, non-physiologic extracellular folate concentrations, the lower-affinity RFC appears responsible for the majority of folate transport (18,19). The precise role of either of these binding proteins and their relative contribution in binding MTX and supplemental folates in patients receiving regular treatment with weekly MTX is presently unclear. It is nevertheless apparent that significant interindividual variations in the activity of these binding proteins, perhaps even variations at different sites within the same individual, could contribute greatly to the relative efficacy and potential toxicity of MTX (20). It is worth noting that leucovorin and folic acid utilize different active transport systems and that both leucovorin and MTX share, and compete for, the same system (Figure 3). Recently, genetic variations in the function of the RFC have been associated with varying response to MTX when studied ex vivo in human leukemia cells (21,22), as would be expected if lesser or greater amounts of MTX entered the cell. The potential role of these single-nucleotide polymorphisms (SNPs) in certain key metabolic steps associated with MTX metabolism will be discussed in greater detail below.

Corticosteroids have been demonstrated to inhibit MTX influx via the RFC in a murine model (23), but it is unclear if this effect occurs in patients receiving low-dose corticosteroids since this has not been studied. The proliferative, or kinetic, state of cells also affects

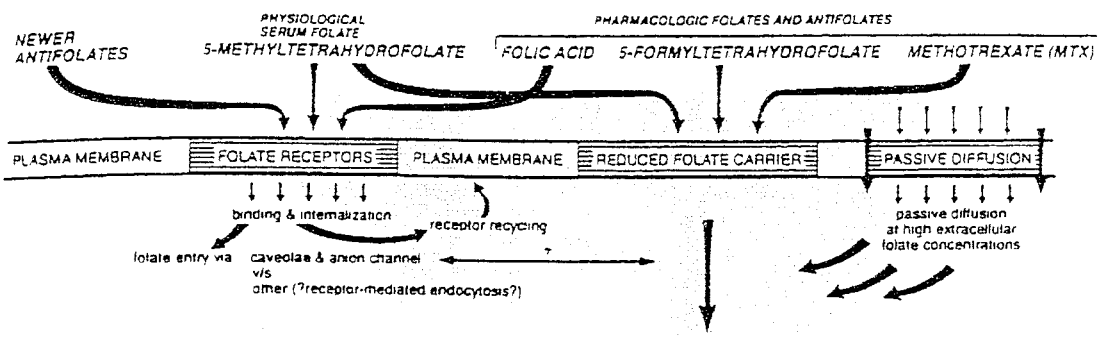

Figure 3. Transport of MTX into the cell. Both MTX and 5-formyltetrahydrofolate (leucovorin) enter the cell via the reduced folate carrier (RFC). Folic acid enters the cell preferentially via the human folate receptor (FR). The FR has a significantly higher affinity for transport of folate than does the RFC. Genetic variability in the RFC has been described. See text for details. Reproduced, with permission, from ref. 9; adapted originally, with permission, from ref. 11.

MTX transport, with rapidly dividing cells taking up MTX more readily with a decreased rate of drug efflux, compared with cells that are either in a stationary phase or are slow growing (24). As described above, activated synovial macrophages from RA synovial tissue exhibit up-regulated FRs, which actually function to enhance MTX transport (15). The resultant inevitable decrease of cellular activity associated with the increased intracellular presence of MTX would therefore lead eventually to decreased uptake of the drug at the FR. That is, long-term, clinically effective administration of MTX could lead to a diminution of its own cellular uptake. This ironic theoretical effect could contribute to the previously described plateau of clinical response to MTX (25).

Efflux of MTX occurs through mechanisms that are somewhat different from uptake and are energy dependent. Recently, multidrug resistance–associated proteins (MRPs), which transport MTX, folic acid, and leucovorin out of cells, have been identified (26). Although the affinity of the MRPs for transport of MTX and folates out of the cell is many times less than that of the RFCs, their high activity in certain cells makes them metabolically relevant. Inhibition of MRPs results in significant accumulation of intracellular MTX. P-glycoprotein is a pump molecule of the MRP class. A recent study demonstrated increased expression of P-glycoprotein in peripheral blood mononuclear cells (PBMCs) of patients with RA who have been judged to have a poor response to MTX, compared with a group with a good response to the drug (27). A breast cancer resistance protein, which results in increased efflux of MTX from tumor cells with resultant resistance to chemotherapy with MTX, has been described (28).

Drugs such as probenecid and prostaglandin A have been reported to decrease MTX efflux (29). Studies on the effects of either agent with the simultaneous use of MTX in patients with rheumatic diseases have not been reported. While probenecid is not used commonly with MTX in RA, it could be used more frequently in patients with other rheumatic diseases in which MTX is also prescribed, including vasculitis. The effect, if any, of the prostaglandin E_1 analog misoprostol on the effectiveness or toxicity of MTX has not been examined in rheumatic disease patients receiving the drug.

The potential contribution of altered uptake of MTX to the emergence of resistance, which could explain the plateau in clinical response to the drug previously noted, has been creatively studied in several cancer cell lines, in investigations using antifolate compounds that do not enter the cell via the active transport system, as well as drugs that competitively displace labeled MTX from the cell membrane (30). It is apparent that resistance to MTX does occur in acute leukemia through the mechanism of altered transport. The potential for altered transport to contribute to MTX resistance has been studied in PBMCs from RA patients who are receiving MTX and was found not to be a factor in diminished response (31). However, RA patients who are homozygous for a mutant SNP of the RFC had a higher probability of improvement in total joint counts, physician assessment of disease activity, and modified Health Assessment Questionnaire scores (32), extending recent observations of the effects of genetic variations in the RFC in patients with leukemia (21,22).

Intracellular transformation

Naturally occurring folates exist within cells as polyglutamates through the action of the enzyme folylpolyglutamyl synthetase (FPGS), which may add up to 6 glutamyl groups in a gamma peptide linkage to the folate substrate (33) (Figure 2). Polyglutamation serves 3 main purposes, as summarized by Chu and Allegra (9): 1) it facilitates the accumulation of intracellular folates in vast excess of the monoglutamate pool, which is freely transportable into and out of cells; 2) it allows selective intracellular retention of these relatively large anionic molecules; and 3) it greatly enhances folate cofactor affinity for several folate-dependent enzymes, including thymidylate synthase and AICAR transformylase (34,35) (Figures 1 and 2).

MTX is also polyglutamated and retained for a long period in the liver of patients with RA (36), as well as in bone marrow myeloid precursors (37) and human fibroblasts (38). The polyglutamation of MTX occurs over 12–24 hours of exposure, at which time most intracellular drug exists in only the polyglutamated form (9). Thus, both physiologic folates and MTX are retained intracellularly by polyglutamation, and MTX will rapidly efflux from the cell in its monoglutamated form.

Of interest, the most avid substrate for FPGS is DHF, followed in descending order by THF, 5-CH_3-THF (found in foods and the major folate species in the circulation), 5-CHO-THF (leucovorin), and then MTX. Reductions of FPGS activity would therefore affect the polyglutamation and retention of MTX to a much greater extent than other naturally occurring folates and could have critical effects on the cellular activity of MTX (9).

It is possible that the inefficient metabolism of 5-CH_3-THF to its polyglutamate form may be responsi-

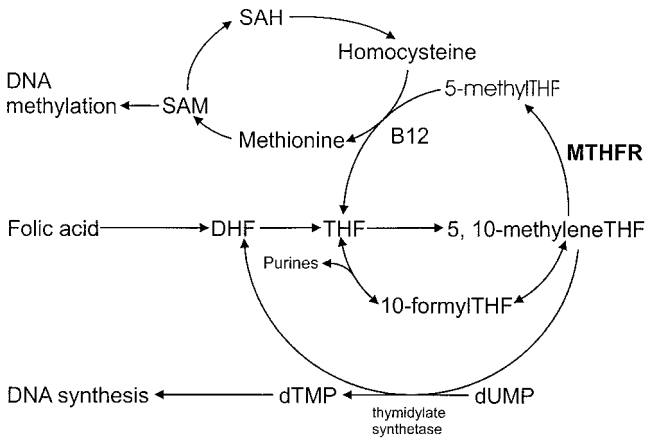

Figure 4. Metabolism of homocysteine to methionine, with MTHFR activity. Methyltetrahydrofolate (methyl-THF), the predominant folate species in the circulation and the form found in food, donates a methyl group to homocysteine to produce methionine in the presence of vitamin B_{12} cofactor. Methionine is then metabolized to S-adenosyl methionine (SAM), which serves as a ubiquitous donator of methyl groups, also contributing to polyamine production, as well as DNA methylation. 5,10-methylene-THF is converted to 5-methyl-THF by the action of the enzyme MTHFR. As discussed in the text, phenotypic differences in the activity of MTHFR have been associated with different responses to MTX, which would be expected if the efficiency of regeneration of 5-methyl-THF varies while patients are receiving MTX. SAH = S-adenosyl homocysteine (see Figure 2 for other definitions).

ble for the folate depletion that occurs with vitamin B_{12} deficiency. Decreased levels of B_{12} would inhibit methionine synthase, the enzyme responsible for the demethylation of 5-CH_3-THF to THF (itself an excellent substrate for FPGS) (9). In the normal state, 5-CH_3-THF contributes a methyl group to homocysteine to form methionine, a reaction which also leads to repletion of THF intracellular pools (Figure 4). In the presence of low B_{12} levels, not only is less methionine generated with resultant accumulation of homocysteine, but less THF is regenerated. Thus, B_{12} deficiency can lead indirectly to intracellular folate depletion as well (Figure 4).

MTX has also been associated with hyperhomocysteinemia because of the diminished activity of methionine synthase. MTX-associated decreases in intracellular stores of the 5-CH_3-THF cofactor, which is required, along with B_{12}, for the conversion of homocysteine to methionine, would also lead to accumulation of homocysteine (39,40) (Figure 4). Since the reduced folates necessary to contribute a methyl group (5-CH_3-THF) in this reaction can be repleted by the activity of 5,10-methylenetetrahydrofolate reductase (MTHFR), the previously noted genetic variations in the efficiency of this enzyme (41) could also contribute to hyperhomocysteinemia (Figure 4). In addition, the inhibition of conversion of homocysteine to methionine caused by MTX-induced reductions in THF would be expected to have an exaggerated effect in individuals with the MTHFR SNP, which results in less efficient repletion of this cofactor (42,43). The subsequent result of decreased levels of methionine (Figure 4) would include potential limiting effects on a myriad of metabolic 1-carbon transfer pathways including polyamine production (44), necessary for cellular growth and replication (45).

As noted above, polyglutamation of MTX appears to be essential for its intracellular activity. The intracellular content of polyglutamates represents a balance between FPGS and γ-glutamyl hydrolase (FPGH) (46) (Figure 2). FPGH removes terminal glutamyl residues and thus returns MTX polyglutamates to their parent monoglutamate form. The monoglutamate form of MTX can easily efflux from the cell, which is not possible for polyglutamated drug. It is thus apparent that the balance between the relative activities of these enzymes is important for deriving the net therapeutic effect of MTX. Levels of intracellular MTX polyglutamates have been measured in circulating red blood cells from patients with RA and were found to correlate with the therapeutic effect of the drug (47,48). It is of interest that MTX exposure can also induce up-regulation of FPGH, which has been implicated as a possible mechanism of folate depletion. FPGH induction would result in the loss of other reduced folate species as well, since they are also retained within intracellular pools in their polyglutamated state, as noted above. Thus MTX-induced up-regulation of FPGH can lead to intracellular folate depletion with resultant toxicities, including neurotoxicity (49).

Several parameters influence a cell's ability to polyglutamate MTX, as summarized by Chu and Allegra (9). Growth factors that enhance cell proliferation, such as insulin, dexamethasone, and estrogen (50), will lead to increased polyglutamation, while deprivation of essential amino acids results in inhibition of polyglutamation (51). Insulin-induced enhanced polyglutamation may provide an explanation as to why patients with insulin-dependent diabetes may be at increased risk for MTX toxicity (52,53). In addition, increasing intracellular folate pools, through exposure of cells to high concentrations of folic acid, leucovorin, or dietary 5-CH_3-THF, will result in a decrease in MTX polyglutamation, because of competition of these folate species for the same enzyme system (9). It is therefore useful to ascertain a dietary history prior to initiation of MTX

Table 1. Possible mechanisms of resistance to MTX in patients with rheumatoid arthritis*

Metabolic event	Effect	Inciting event	References
DHFR gene duplication	Increased DHFR	MTX treatment	59–65
Increased translation of DHFR mRNA	Increased DHFR	MTX binding to DHFR	31, 66, 67
Increased DHFR gene expression	Increased DHFR	Cellular stress, hypoxia, UV radiation, environmental carcinogen	9, 69, 70, 71
Genetic variation in MTX metabolism	Altered intracellular MTX concentration	MTX exposure	21, 22, 27, 30, 32, 42, 43, 48, 55, 73

* MTX = methotrexate; DHFR = dihydrofolate reductase; UV = ultraviolet.

treatment. Individuals who consume foods high in folate or who routinely ingest multivitamins may be at less risk for MTX toxicity and may also need a higher dosage of MTX for achievement of the desired therapeutic effect.

When MTX is used for treatment of malignancy, decreased polyglutamation is observed in normal versus malignant cells (54). In a similar manner, the selective salvage of normal versus malignant cells with the use of leucovorin rescue after high-dose MTX treatment is possible because of the differential effects of MTX on normal cells, with a lower metabolic rate of activity, than on the more metabolically active malignant tissue.

The differential effects of MTX in inflammatory versus normal tissue have not been studied. It is possible, however, that the relative preservation of the therapeutic effects of MTX usually observed after addition of either folic acid or leucovorin to the regimen in MTX-treated patients with RA is due to naturally occurring differential rates of polyglutamation in normal (resting) versus inflammatory (activated) cell populations, with the former needing less reduced folate to "rescue" than the latter.

The observation that the therapeutic effect of MTX is retained in spite of folate supplementation is often invoked in support of the theory that the mechanism of action of MTX is independent of folate inhibition. However, as noted, this line of reasoning fails to consider the differential effects of both MTX and folate repletion on normal versus more metabolically active cell populations, which could be responsible for sustaining disease. Differential effects of MTX on cells with varying metabolic activity within the same individual could provide an explanation as to why folate supplementation does not remove most of the therapeutic value of the drug. Normal resting cell populations would simply require less additional folate to block MTX activity.

In addition to increasing cellular retention, polyglutamates of MTX enhance its direct inhibitory effect on other target enzymes, including DHFR, as well as AICAR transformylase and GAR transformylase (Figure 1), two key enzymes in the purine metabolic pathway inhibited by MTX (9).

Does MTX resistance occur in patients with RA?

Resistance to MTX is well described for cancer patients and may involve several possible mechanisms (28,55–57). An increase in the level of DHFR protein is frequently found, with no change in the affinity of the enzyme for MTX (31,58). In murine and human leukemic cells, the MTX-induced increased DHFR activity may be associated with reduplication of the DHFR gene (59,60) (Table 1), also demonstrated in a Jurkat T cell line (61). These MTX-induced increases in gene number may persist for generations in tumor lines (62). Gene reduplication may take the form of homogeneously staining regions on chromosomes or nonintegrated pieces of DNA known as double-minute chromosomes, which are unevenly distributed during cell division (63,64). There is evidence that gene amplification is the initial stage in low-level drug-resistant cells (9), while homogeneously staining regions contain multiple gene copies (65). A third mechanism of gene amplification occurs with the formation of submicroscopic extrachromosomal elements, termed amplisomes, containing extrachromosomal DHFR genes (9). It should be noted that gene amplification in RA patients receiving long-term weekly MTX treatment has been sought and was not found (31). Increased DHFR protein from PBMCs was, however, observed in that investigation (31). It is possible that gene amplification occurred through amplisomes; this mechanism was not investigated.

Other mechanisms for MTX resistance include acute increases in cellular DHFR content due to changes in the level of messenger RNA (mRNA) translation, with no change in the level of DHFR mRNA or DHFR gene copy number following MTX exposure (66,67) (Table 1). It is fascinating that recombinant human DHFR specifically binds to its corresponding

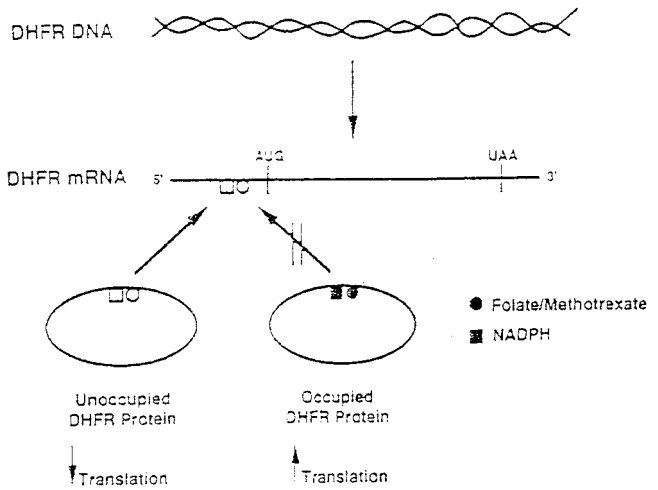

Figure 5. Binding of folate protein to dihydrofolate reductase (DHFR) mRNA. Binding of DHFR protein to DHFR mRNA provides a feedback loop for inhibition of further production. The forward reaction is inhibited by binding of DHFR to both folate and methotrexate (MTX), and is also inhibited in the presence of NADPH. Thus, increased production of DHFR protein would be expected to occur in the presence of both supplemental folate and MTX because of a diminished feedback of unbound DHFR on mRNA. See text for details of possible association with resistance to MTX. Reproduced, with permission, from ref. 9.

DHFR mRNA (68) (Figure 5). It therefore appears that a translational regulatory system of direct binding of DHFR mRNA to its protein provides an autoregulatory mechanism underlying the control of DHFR expression (9). It was this mechanism of binding of message to DHFR mRNA that was hypothesized to explain the observation of an increased level of DHFR in PBMCs from RA patients receiving MTX (31). The relationship between increased cellular levels of DHFR and clinical response to MTX in RA patients has been examined in only this one small study and warrants further investigation.

As noted by Chu and Allegra (9), the presence of either excess MTX or excess dihydrofolate could prevent DHFR protein from performing its normal autoregulatory (feedback) function (because of binding to DHFR, as depicted in Figure 5), thereby allowing for increased DHFR protein synthesis. It is the ability to regulate DHFR synthesis at the translational level that allows normal cellular function to be maintained in the setting of an acute cellular stress and "represents a unique mechanism whereby cells can react to and overcome the inhibitory effects of MTX and antifolate analogues" (9).

There is also a body of evidence indicating that cells respond to a variety of stressors, including hypoxia (69), ultraviolet irradiation (70), and chemotherapeutic agents (71), by amplifying DHFR gene expression, with resultant increases in DHFR protein (Table 1). It has been hypothesized that a variety of environmental carcinogens could actually contribute to the phenomenon of MTX resistance by inducing gene amplification (9). The plateau of clinical response to MTX that occurs after 6 months of treatment in patients with RA (25) may be related to some, all, or none of these phenomena of resistance. Given the "cornerstone" placement of the drug at this time, they warrant further study.

Metabolism and cytotoxicity

A more powerful factor than drug concentration as a determinant of cellular toxicity is actual duration of exposure to MTX in concentrations that exceed threshold levels necessary for cellular cytotoxicity. It is generally accepted that serum concentrations of MTX that exceed 0.05 μM for >24 hours result in these cytotoxic effects. General peak values of ~0.3–0.8 μM, or slightly higher, are common after weekly dosing, with values falling to <0.05 μM by 24 hours after a single dose of MTX (for review of typical serum MTX concentrations achieved in vivo in RA, see ref. 36). Because MTX is excreted entirely via the kidneys, it follows that any compromise in renal function that prolongs the retention of the drug at serum levels that exceed 0.05 μM 24 hours postadministration may be associated with clinical toxicity in certain rapidly dividing cell populations. Actively dividing cells in the S-phase are the most vulnerable. With longer duration of exposure to MTX, more rapidly dividing cells will enter the S-phase, and more cytotoxicity will occur (72). These first toxicities in response to MTX are thus likely to be seen in the epithelial lining layers of the oral mucosa and gastrointestinal (GI) tract, bone marrow cells, and testicular tissue involved in spermatogenesis (9).

Another determinant of MTX-induced cellular toxicity is the level of 5-CH_3-THF in the circulation, which can readily reverse MTX toxicity (9). Both dietary sources and the activity of MTHFR determine the levels of 5-CH_3-THF (9) (Figures 2 and 4). As noted above, well-described genetic variations in an individual's ability to regenerate 5-CH_3-THF may independently contribute to both the efficacy and the toxicity of MTX (42,43). MTHFR genotypes in RA patients receiving MTX have been examined, and the results correlated with response to MTX (73). Other genetically deter-

mined variations in the ability to absorb and metabolize MTX within a cell, including the activity of the RFC and the AICAR transformylase enzyme, may also likely account for some commonly seen differences in therapeutic effects (32) (Table 1).

Absorption

MTX is absorbed from the GI tract by a saturable active transport system (74). Absorption of MTX administered orally at 7.5 mg/week is roughly equivalent to that of parenterally administered drug, but absorption of oral MTX drops off by as much as 30% as the weekly dose reaches 15 mg or greater (75). The decreased absorption of oral MTX at dosage levels commonly used in the treatment of RA explains the incremental improvement frequently seen when patients are switched from the oral to parenteral route of administration. Food does not affect MTX absorption, although milk may inhibit absorption (76).

Orally administered MTX is absorbed via the GI tract and passes through the portal vein to the liver. While parenterally administered MTX would also enter the liver via the hepatic artery, the relative potential for diminished hepatotoxicity with the parenteral versus the oral route of administration would seem to favor the former route, although this has not been prospectively studied in RA patients receiving long-term MTX treatment. A recent retrospective study did demonstrate a lesser frequency of elevations of transaminase levels into the abnormal range when the drug was administered parenterally, versus orally, in the same individuals (77). Long-term administration of oral MTX to patients with RA is associated with reduced hepatic folate stores, which may be repleted with only a few days of orally administered leucovorin (36). In spite of this observation, the relationship between hepatic cellular folate depletion and MTX-induced liver toxicity is imperfectly understood.

Distribution

MTX will accumulate in third space fluid, which can serve as a reservoir for redistribution into the circulation long after dosing (78). Therefore, MTX must be used with extreme caution in patients with either pleural effusions or ascites. Since pleural reactions, including pleural effusions, can also be a manifestation of MTX-induced pulmonary toxicity (79), this mechanism of exaggerated MTX effect could be induced by the drug itself. Unexpectedly high levels of MTX have also been observed in patients with bladder cancer who have undergone ileal conduit surgery (80), due to enhanced absorption through the newly fashioned intestinal conduit.

Renal excretion

The majority of MTX is excreted in the urine in the first 12 hours after administration, with renal excretion approaching 100% in patients with normal kidney function (81). MTX clearance may exceed renal clearance (82,83), probably due to active secretion of MTX in the proximal renal tubule with subsequent reabsorption in the distal tubule, as observed in animal models (84). MTX elimination cannot be predicted solely on the basis of creatinine clearance; tubular excretion contributes a variable effect in different patients.

MTX has been reported to cause slight decreases in clearance of creatinine in RA patients during the first 6 months of treatment, but the precise mechanism of this effect is unclear (85). In patients who have received long-term MTX treatment, the effect of MTX itself on renal function must be considered when other causes for a reduced serum creatinine level have been ruled out.

Excretion of MTX is inhibited by weak organic acids such as aspirin, nonsteroidal antiinflammatory drugs (NSAIDs), piperacillin, penicillin G, and probenicid (9). Cephalosporins may also inhibit renal excretion, probably by competition for tubular secretion (86). A single recent publication indicates that hydroxychloroquine appears to enhance the effects of MTX by increasing the area under the curve for MTX concentration time by an average of 65% (87). The mechanism of the increase was thought to be either a reduction in MTX clearance or an increase in the active tubular reabsorption of MTX in the presence of hydroxychloroquine. If confirmed, it is possible that this effect accounts for some of the success of the addition of hydroxychloroquine and sulfasalazine to an MTX regimen (3). Simultaneous folic acid administration will also block MTX reabsorption at the distal tubule (9).

While conflicting reports of the possible inhibitory effect of NSAIDs on MTX excretion have been published, it appears that inhibitory effects on MTX elimination could become clinically relevant at the typical higher weekly maintenance MTX dosage range used in the treatment of RA (88), but not at the lower doses commonly used to begin treatment. It is thus relevant to reexamine serum creatinine levels, and to remain vigilant for MTX toxicity, whenever NSAIDs are added to

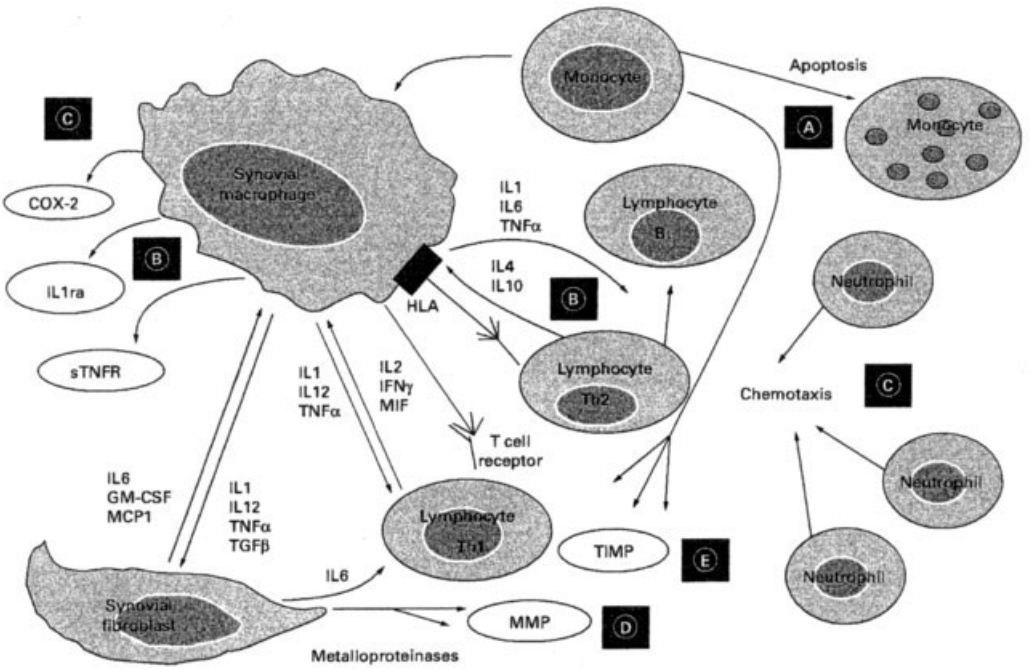

Figure 6. Antiinflammatory effects of methotrexate (MTX) at the level of the synovium in rheumatoid arthritis. **A,** MTX reduces growth of monocytes and increases their apoptosis. **B,** MTX decreases interleukin-1 (IL-1) and IL-6 secretion and increases IL-1 receptor antagonist (IL-1Ra) production. Also, MTX increases IL-4 and IL-10 gene expression and decreases gene expression of proinflammatory Th1 cytokines (IL-2 and interferon-γ [IFNγ]). **C,** MTX exerts indirect inhibitory effects on production of cyclooxygenase 2 (COX-2) synthesis and neutrophil chemotaxis. **D** and **E,** MTX exerts indirect inhibitory effects, through cytokine modulation, on matrix metalloproteinase (MMP) production and stimulates tissue inhibitor of metalloproteinases (TIMP). TNFα = tumor necrosis factor α; sTNFR = soluble TNF receptor; GM-CSF = granulocyte–macrophage colony-stimulating factor; MCP-1 = monocyte chemotactic protein 1; TGFβ = transforming growth factor β; MIF = macrophage migration inhibitory factor. Reproduced, with permission, from ref. 95.

the regimen, or changed, in patients receiving a stable maintenance dose of weekly MTX. Many NSAIDs with an aromatic group and carboxylic acid side chain, including ibuprofen, naproxen, sulindac, mefenamic acid, aspirin, and indomethacin, may actually be able to directly inhibit both DHFR and AICAR transformylase (89). These NSAIDs would thus theoretically have dual mechanisms for increasing the effects, and toxicity, of MTX.

Although this is not generally well recognized, detailed population studies performed after extensive pharmacokinetic testing of MTX in patients with RA show a decreased clearance of the drug in female compared with male patients, which persists after correction for creatinine clearance and body weight (90). The cause of the decreased clearance is unclear. It is nevertheless apparent that women may be at increased risk for MTX toxicity.

Toxicity

Specific MTX-associated toxicities have been extensively reported previously and will not again be reviewed here. It is, however, appropriate to consider the varying sensitivities of different cell populations to the toxic effects of MTX, a subject that is rarely, if ever, addressed in publications within the rheumatology literature describing the toxicities of the drug. It is known that the oral and intestinal epithelium are more sensitive to the effects of MTX, with the development of mucositis, than is the bone marrow (9). Concentrations of MTX that produce mucositis are rarely associated with marrow suppression. The threshold plasma concentration of MTX required to inhibit DNA synthesis in bone marrow has been estimated to be 10 n*M*, whereas GI epithelium is inhibited at a plasma concentration of 5 n*M* (91). The greater sensitivity of GI epithelium to MTX toxicity may

also be due to greater accumulation and persistence of MTX in intestinal epithelium as opposed to bone marrow (92).

Myelosuppression and mucositis are usually reversed within 2 weeks, unless drug excretion mechanisms are severely impaired. In patients with compromised renal function, even small doses may result in cytotoxic blood levels for up to 3–5 days (9). The extent to which mucositis and GI toxicity have been virtually eliminated by the addition of folic acid to the treatment regimen in patients who may still encounter occasional marrow suppression (93,94) provides evidence of the distinctive susceptibility of these specific tissues to MTX.

Mechanism of action

Methotrexate undoubtedly works in a variety of ways in RA, as well as in other rheumatic and inflammatory conditions, as summarized by Cutolo et al (95) and in Figure 6. As demonstrated by Cronstein (96), MTX treatment results in increased release of adenosine, which is associated with several antiinflammatory and immunosuppressive effects (Figure 1). The potential salutary effects of MTX in dampening the immune/inflammatory process are extraordinarily diverse, perhaps accounting for its utility in such a wide range of inflammatory conditions and in combination with such a variety of other interventions.

Summary

In spite of the fact that MTX has become the most widely prescribed DMARD, clinical prescribing behaviors are often habitual and frequently reflect patterns learned at a time when less was understood about the metabolism of the drug, reasons for toxicity, or possible means of overcoming resistance. Since most of the insights regarding the use of MTX and its effects on cellular metabolism are derived from the oncology literature, it is apparent that rheumatologists are in the debt of these researchers, and that similar observations should be sought when the drug is used in the long-term treatment of patients with rheumatic diseases. Rheumatologists are usually well informed about the many clinical issues associated with the use of MTX, but clinical decisions may frequently be enhanced by a sound understanding of the complexities of the drug's cellular metabolism. These insights can contribute to more effective, safer, and more satisfying practice patterns.

ACKNOWLEDGMENTS

The author wishes to thank John Galivan, PhD, Erasmus Schneider, PhD, and Thierry Dervieux, PharmD, PhD, for their thoughtful review and suggestions, which have contributed significantly to the production of the manuscript in its final form.

REFERENCES

1. Farber S, Diamond LK, Mercer RD, Sylvester RF, Wolff JA. Temporary remissions in acute leukemia in children produced by folic antagonist 4-amethopteroylglutamic acid (aminopterin). N Engl J Med 1948;238:787–93.
2. Tugwell P, Pincus T, Yocum D, Stein M, Gluck O, Kraag G, et al, The Methotrexate-Cyclosporine Combination Study Group. Combination therapy with cyclosporine and methotrexate in severe rheumatoid arthritis. N Engl J Med 1995;333:137–41.
3. O'Dell J, Haire CE, Erikson N, Drymalski W, Palmer W, Eckhoff PJ, et al. Treatment of rheumatoid arthritis with methotrexate alone, sulfasalazine and hydroxychloroquine, or a combination of all three medications. N Engl J Med 1996;334:1287–91.
4. Weinblatt ME, Kremer JM, Bankhurst AD, Bulpitt KJ, Fleischmann RM, Fox RI, et al. A trial of etanercept, a recombinant tumor necrosis factor:Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. N Engl J Med 1999;340:253–9.
5. Lipsky PE, van der Heijde DM, St. Clair EW, Furst DE, Breedveld FC, Kalden JR, et al. Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. N Engl J Med 2000;343:1594–602.
6. Kremer JM, Genovese MC, Cannon GW, Caldwell JR, Cush JJ, Furst DE, et al. Concomitant leflunomide therapy in patients with active rheumatoid arthritis despite stable doses of methotrexate: a randomized comparison of efficacy, safety, and tolerability compared to methotrexate alone. Ann Intern Med 2002;37:726–33.
7. Weinblatt ME, Keystone EC, Furst DE, Moreland LW, Weisman MH, Birbara CA, et al. Adalimumab, a fully human anti–tumor necrosis factor α monoclonal antibody, for the treatment of rheumatoid arthritis in patients taking concomitant methotrexate: the ARMADA trial. Arthritis Rheum 2003;48:35–45.
8. Kremer JM, Westhovens R, Leon M, Di Georgio E, Alten R, Steinfeld S, et al. Treatment of rheumatoid arthritis by prevention of T cell activation with fusion protein CTLA4Ig. N Engl J Med 2003;349:1907–15.
9. Chu E, Allegra C. Antifolates. In: Chabner BA, Longo DL, editors. Cancer chemotherapy and biotherapy. 2nd ed. Philadelphia: Lippincot-Raven; 1996. p. 109–47.
10. Matherly LH, Wong SC, Angeles SM, Taub JW, Smith GK. Distribution of the reduced folate carrier (RFC) versus the high affinity membrane folate binding protein (mFBP) in human tumors and tissues. Proc Am Assoc Cancer Res 1994;35:307–15.
11. Antony AC. The biological chemistry of folate receptors. Blood 1992;79:2807–20.
12. Wang J, Ginning W, Delley KM, Ratnam M. Evidence for segregation of heterologous GPI-anchored proteins into separate lipid rafts within the plasma membrane. J Membr Biol 2002;189:35–43.
13. Ross JF, Chaudhuri PK, Ratnam M. Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines: physiologic and clinical implications. Cancer 1994;73:2432–43.
14. Matherly LH, Goldman D. Membrane transport of folates. Vitam Horm 2003;66:403–56.
15. Nakashima-Matsushita N, Homma T, Yu S, Matsuda T, Sunahara

N, Nakamura T, et al. Selective expression of folate receptor β and its possible role in methotrexate transport in synovial macrophages from patients with rheumatoid arthritis. Arthritis Rheum 1999;42: 1609–16.
16. Turk MJ, Breur GJ, Widmer WR, Paulos CM, Xu LC, Grote LA, et al. Folate-targeted imaging of activated macrophages in rats with adjuvant-induced arthritis. Arthritis Rheum 2002;46:1947–55.
17. Kane MA, Portillo RM, Elwood PC, Antony AC, Kolhouse JF. The influence of extracellular folate concentration on methotrexate uptake by human KB cells: partial characterization of a membrane-associated methotrexate binding protein. J Biol Chem 1986;261:44–9.
18. Antony AC, Kane MA, Portillo RM, Elwood PC, Kolhouse JF. Studies of the role of a particulate folate-binding protein in the uptake of 5-methyltetrahydrofolate by cultured human KB cells. J Biol Chem 1985;260:14911–7.
19. Kamen BA, Capdevila A. Receptor-mediated folate accumulation regulated by the cellular folate content. Proc Natl Acad Sci U S A 1986;83:5983–7.
20. Weitman SD, Weinberg AG, Coney LR, Zurawski VR, Jennings DS, Kamen BA. Cellular localization of the folate receptor: potential role in drug toxicity and folate homeostasis Cancer Res 1992;52:6708–11.
21. Whetstine JR, Gifford AJ, Witt T, Liu XY, Flatley RM, Norris M, et al. Single nucleotide polymorphisms in the human reduced folate carrier: characterization of a high-frequency G/A variant at position 80 and transport properties of the His(27) and Arg(27) carriers. Clin Cancer Res 2001;7:3416–22.
22. Rothem L, Aronheim A, Assaraf YG. Alterations in the expression of transcription factors and the reduced folate carrier as a novel mechanism of antifolate resistance in human leukemia cells. J Biol Chem 2002;278:8935–41.
23. Zager RF, Frisby SA, Oliverio VT. The effects of antibiotics and cancer chemotherapeutic agents on cellular transport and antitumor activity of methotrexate in L1210 murine leukemia. Cancer Res 1973;33:1670–6.
24. Chello PL, Sirotnak FM, Dorick DM. Alterations in the kinetics of methotrexate transport during growth of L1210 murine leukemia cells in culture. Mol Pharmacol 1980;18:274–80.
25. Kremer JM, Lee JK. The safety and efficacy of the use of methotrexate in long-term therapy for rheumatoid arthritis. Arthritis Rheum 1986;29:822–31.
26. Chen ZS, Lee K, Walther S, Raftogianis RB, Kuwano M, Zeng H, et al. Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABC4): MRP4 is a component of the methotrexate efflux system. Cancer Res 2002;62:3144–50.
27. Ranganathan P, Eisen S, Yokoyama WM, McLeod HL. Will pharmacogenetics allow better prediction of methotrexate toxicity and efficacy in patients with rheumatoid arthritis? Ann Rheum Dis 2003;62:4–9.
28. Volk S, Farley KM, Wu Y, Li F, Robey RW, Schneider E. Expression of wild-type breast cancer resistance protein mediates methotrexate resistance. Cancer Res 2002;62:5035–40.
29. Sirotnak FM, Moccio DM, Hancock CH, Young CW. Improved methotrexate therapy of murine tumors obtained by probenecid-mediated pharmacocological modulation at the level of membrane transport. Cancer Res 1981;41:3944–9.
30. Trippett T, Schlemmer S, Elisseyeff Y, Goker E, Wachter M, Steinherz P, et al. Defective transport as a mechanism of acquired resistance to methotrexate in patients with acute lymphoblastic leukemia. Blood 1992;80:1158–62.
31. Rodenhuis S, Kremer JM, Bertino JR. Increase of dihydrofolate reductase in peripheral blood lymphocytes of rheumatoid arthritis patients treated with low-dose oral methotrexate. Arthritis Rheum 1987;30:369–74.
32. Dervieux T, Orentas Lein D, Park G, Barham R, Smith K, Walsh M, et al. Single nucleotide polymorphisms in the folate/purine synthesis pathway predict methotrexate's effects in rheumatoid arthritis [abstract]. Arthritis Rheum 2003;48 Suppl 9:S438.
33. McGuire JJ, Coward JK. Pteroylpolyglutamates: biosynthesis, degradation, and function. In: Blakley RL, Benkovic SJ, editors. Folates and pterins. Vol. 1. Chemistry and biochemistry of folates. New York: John Wiley; 1984. p. 135–90.
34. Kumar P, Kisliuk RL, Gaumont Y, Freisheim JH, Nair MG. Inhibition of human dihydrofolate reductase by antifolyl polyglutamates. Biochem Pharmacol 1989;38:541–3.
35. Morrison PF, Allegra CJ. Folate cycle kinetics in human breast cancer cells. J Biol Chem 1989;264:10552–66.
36. Kremer JM, Galivan J, Streckfuss A, Kamen B. Methotrexate metabolism analysis in blood and liver of rheumatoid arthritis patients: association with hepatic folate deficiency and formation of polyglutamates. Arthritis Rheum 1986;29:832–5.
37. Koizumi S, Curt GA, Fine RL, Griffin JD, Chabner BA. Formation of methotrexate polyglutamates in purified myeloid precursor cells from normal human bone marrow. J Clin Invest 1985;75: 1008–14.
38. Rosenblatt DS, Whitehead VM, Dupont MM, Vuchich MJ, Vera N. Synthesis of methotrexate polyglutamates in cultured human cells. Mol Pharmacol 1978;14:210–4.
39. Morgan SL, Baggott JE, Refsum H, Ueland PM. Homocysteine levels in patients with rheumatoid arthritis treated with low-dose methotrexate. Clin Pharmacol Ther 1991;50:547–56.
40. Morgan SL, Baggott JE, Lee JY, Alarcon GS. Folic acid supplementation prevents deficient blood folate levels and hyperhomocysteinemia during long-term, low dose methotrexate therapy for rheumatoid arthritis: implications for cardiovascular disease prevention. J Rheumatol 1998;25:441–6.
41. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111–3.
42. Van Ede AE, Laan RF, Blom HJ, Huizinga TW, Haagsma CJ, Giesendorf BA, et al. The C677T mutation in the methylenetetrahydrofolate reductase gene: a genetic risk factor for methotrexate-related elevation of liver enzymes in rheumatoid arthritis patients. Arthritis Rheum 2001;44:2525–30.
43. Ulrich CM, Yasui Y, Storb R, Schubert MM, Wagner JL, Bigler J, et al. Pharmacogenetics of methotrexate: toxicity among marrow transplantation patients varies with the methylenetetrahydrofolate reductase C677T polymorphism. Blood 2001;98:231–4.
44. Nesher G, Moore TL. The in vitro effects of methotrexate on peripheral blood mononuclear cells: modulation by methyl donors and spermidine. Arthritis Rheum 1990;33:954–9.
45. Abraham AK, Pihl A. Role of polyamines in macromolecular synthesis. Trends Biochem Sci 1981;6:106–7.
46. Galivan J, Johnson T, Rhee M, McGuire JJ, Priest D, Kesevan V. The role of folylpolyglutamate synthase and γ-glutamyl hydrolase in altering cellular folyl- and antifolylpolyglutamates. Adv Enzyme Regul 1987;26:147–55.
47. Angelis-Stoforidis P, Vajda FJ, Christophidis N. Methotrexate polyglutamate levels in circulating erythrocytes and polymorphs correlate with clinical efficacy in rheumatoid arthritis. Clin Exp Rheumatol 1999;17:313–20.
48. Dervieux T, Orentas Lein D, Park G, Marcelletti J, Meyer G, Smith K, et al. Methotrexate polyglutamate concentrations in red blood cells correlate with disease activity and clinical response to methotrexate in rheumatoid arthritis [abstract]. Arthritis Rheum 2003;48 Suppl 9:S135.
49. Schlemmer SR, Sirotnak FM. Retentiveness of methotrexate polyglutamates in cultured L1210 cells: evidence against a role for mediated plasma membrane transport outward. Biochem Pharmacol 1993;45:1261–6.
50. Galivan J, Rhee MS. Insulin-dependent suppression in glutamyl

hydrolase activity and elevated cellular methotrexate polyglutamates. Biochem Pharmacol 1995;50:1959–63.
51. Jolivet J, Cole DE, Holcenberg JS, Poplack DG. Prevention of methotrexate cytotoxicity by asparaginase inhibition of methotrexate polyglutamate formation. Cancer Res 1985;45:217–20.
52. Kremer JM, Alarcon GS, Lightfoot RW Jr, Willkens RF, Furst DE, Williams HJ, et al. Methotrexate for rheumatoid arthritis: suggested guidelines for monitoring liver toxicity. Arthritis Rheum 1994;37:316–28.
53. Alarcon GS, Kremer JM, Macaluso M, Weinblatt ME, Cannon GW, Palmer WR, et al, Methotrexate-Lung Study Group. Risk factors associated with lung injury in methotrexate-treated rheumatoid arthritis patients: a multicenter, case-control study. Ann Intern Med 1997;127:356–64.
54. Fabre I, Fabre G, Goldman ID. Polyglutamylation, an important element in methotrexate cytotoxicity and selectivity in tumor versus murine granulocytic progenitor cells in vitro. Cancer Res 1984;44:3190–5.
55. Bertino JR. Ode to methotrexate: Karnofsky memorial lecture. J Clin Oncol 1993;11:5–14.
56. Rots MG, Pieters R, Kaspers GJ, Veerman AJ, Peters GJ, Jansen G. Classification of ex vivo methotrexate resistance in acute lymphoblastic and myeloid leukaemia. Br J Haematol 2000;110: 791–800.
57. Zhao R, Goldman ID. Resistance to antifolates. Oncogene 2003; 22:7431–57.
58. Friedkin M, Crawford E, Humphreys SR, Goldin A. The association of increased dihydrofolate reductase with amethopterin resistance in mouse leukemia. Cancer Res 1962;22:600–6.
59. Milbrandt JD, Heintz NH, White WC, Rothman SM, Hamlin JL. Methotrexate-resistant Chinese hamster ovary cells have amplified a 135-kilobase-pair region that includes the dihydrofolate reductase gene. Proc Natl Acad Sci U S A 1981;78:6043–7.
60. Domin BA, Grill SP, Cheng Y. Establishment of dihydrofolate reductase from a methotrexate-resistant human cell line and relationship between dihydrofolate reductase levels and gene copy. Cancer Res 1983;43:2155–8.
61. Hall MJ, Lawrence DA, Lansiedel JC, Walsh AC, Comstock LL, Kremer JM. Long-term exposure to methotrexate induces immunophenotypic changes, decreased methotrexate uptake and increased dihydrofolate gene copy number in Jurkat T cells. Int J Immunopharmacol 1997;19:709–20.
62. Bertino JR, Donohue DR, Gabrio BW, Silber R, Alenty A, Meyer M, et al. Increased level of dihydrofolate reductase in leukocytes of patients treated with amethopterin. Nauchni Tr Vissh Med Inst Sofiia 1962;193:140–2.
63. Haber DA, Schimke RT. Unstable amplification of an altered dihydrofolate reductase gene associated with double-minute chromosomes. Cell 1981;26:355–62.
64. Brown PC, Beverley SM, Schimke RT. Relationship of amplified dihydrofolate reductase genes to double minute chromosomes in unstably resistant mouse fibroblast cell lines. Mol Cell Biol 1981;1:1077–83.
65. Meltzer PS, Cheng YC, Trent JM. Analysis of dihydrofolate reductase gene amplification in a methotrexate-resistant human tumor cell line. Cancer Genet Cytogenet 1985;17:289–300.
66. Domin BA, Grill SP, Bastow KF, Cheng YC. Effect of methotrexate on dihydrofolate reductase activity in methotrexate-resistant human KB cells. Mol Pharmacol 1982;21:478–82.
67. Bastow KF, Prabhu R, Cheng YC. The intracellular content of dihydrofolate reductase: possibilities for control and implications for chemotherapy. Adv Enzyme Regul 1984;22:15–26.
68. Chu E, Takimoto CH, Voeller D, Grem JL, Allegra CJ. Specific binding of human dihydrofolate reductase protein to dihydrofolate reductase messenger RNA in vitro. Biochemistry 1993;32: 4756–60.
69. Kleinberger T, Etkin S, Lave S. Carcinogen-mediated methotrexate resistance and dihydrofolate reductase amplification in Chinese hamster cells. Mol Cell Biol 1986;6:1958–64.
70. Sharma RC, Schimke RT. Enhancement of the frequency of methotrexate resistance by γ-radiation in Chinese hamster ovary and mouse 3T6 cells. Cancer Res 1989;49:3861–6.
71. Rice GC, Ling V, Schimke RT. Frequencies of independent and simultaneous selection of Chinese hamster cells for methotrexate and doxorubicin (Adriamycin) resistance. Proc Natl Acad Sci U S A 1987;84:9261–4.
72. Pinedo HM, Zaharko DS, Bull J, Chabner BA. The relative contribution of drug concentration and duration of exposure to mouse bone marrow toxicity during continuous methotrexate infusion. Cancer Res 1977;37:445–50.
73. Urano W, Taniguchi A, Yamanaka H, Tanaka E, Nakajima H, Matsuda Y, et al. Polymorphisms in the methylenetetrahydrofolate reductase gene were associated with both the efficacy and toxicity of methotrexate used for the treatment of rheumatoid arthritis, as evidenced by single locus and haplotype analyses. Pharmacogenetics 2002;12:183–90.
74. Chungi VS, Bourne DW, Dittert LW. Drug absorption. VIII. Kinetics of GI absorption of methotrexate J Pharm Sci 1978;67: 560–1.
75. Hamilton RA, Kremer JM. Why intramuscular methotrexate works better than oral drug in patients with rheumatoid arthritis. Br J Rheumatol 1997;36:86–90.
76. Hamilton RA, Kremer JM. The effect of food on methotrexate absorption. J Rheumatol 1995;22:2072–7.
77. Wegrzyn J, Adeleine P, Miossec P. Better efficacy of methotrexate administered by intramuscular injections versus oral route in patients with rheumatoid arthritis. Ann Rheum Dis. In press.
78. Chabner BA, Stoller RG, Hande K, Jacobs S, Young RC. Methotrexate disposition in humans: case studies in ovarian cancer and following high-dose infusion. Drug Metab Rev 1978;8:107–17.
79. Kremer JM, Alarcon GS, Weinblatt ME, Kaymakcian MV, Macaluso M, Cannon GW, et al. Clinical, laboratory, radiographic and histopathologic features of methotrexate-associated lung injury in patients with rheumatoid arthritis: a multicenter study with literature review. Arthritis Rheum 1997;40:1829–37.
80. Fossa SD, Heilo A, Bormer O. Unexpectedly high serum methotrexate levels in cystectomized bladder cancer patients with an ileal conduit treated with intermediate doses of the drug. J Urol 1990;143:498–501.
81. Calvert AH, Bondy PK, Harrap KR. Some observations on the human pharmacology of methotrexate. Cancer Treat Rep 1977; 61:1647–56.
82. Hande KR, Balow JE, Drake JC, Rosenberg SA, Chabner BA. Methotrexate and hemodialysis. Ann Intern Med 1977;87:495–6.
83. Monjanel S, Rigault JP, Cano JP, Carcassonne Y, Favre R. High-dose methotrexate: preliminary evaluation of a pharmacokinetic approach. Cancer Chemother Pharmacol 1979;3:189–96.
84. Huang KC, Wenczak BA, Liu YK. Renal tubular transport of methotrexate in the rhesus monkey and dog. Cancer Res 1979;39: 4843–8.
85. Kremer JM, Petrillo GF, Hamilton RA. Pharmacokinetics and renal function in patients with rheumatoid arthritis receiving a standard dose of oral weekly methotrexate over a period of 24 months: association with significant decreases in creatinine clearance and renal clearance of the drug. J Rheumatol 1995;22:38–40.
86. Iven H, Brasch H. The effects of antibiotics and uricosuric drugs on the renal elimination of methotrexate in rabbits. Cancer Chemother Pharmacol 1990;26:139–43.
87. Carmichael SJ, Beal J, Day RO, Tett SE. Combination therapy with methotrexate and hydroxychloroquine for rheumatoid arthritis increases exposure to methotrexate. J Rheumatol 2002;29: 2077–83.
88. Kremer JM, Hamilton RA. The effects of nonsteroidal antiinflammatory drugs on methotrexate (MTX) pharmacokinetics: impair-

ment of renal clearance of MTX at weekly maintenance doses but not at 7.5 mg. J Rheumatol 1995;22:2072–7.
89. Baggott JE, Morgan SL, Ha T, Vaughn WH, Hine RJ. Inhibition of folate-dependent enzymes by non-steroidal anti-inflammatory drugs. Biochem J 1992;282:197–202.
90. Godfrey C, Sweeney K, Miller K, Hamilton R, Kremer J. The population pharmacokinetics of long-term methotrexate in rheumatoid arthritis. Br J Clin Pharmacol 1998;46:369–76.
91. Chabner BA, Young RC. Threshold methotrexate concentration for in vivo inhibition of DNA synthesis in normal and tumorous target tissues. J Clin Invest 1973;52:1804–11.
92. Sirotnak F, Moccio DM. Pharmacokinetic basis for differences in methotrexate sensitivity of normal proliferative tissues in the mouse. Cancer Res 1980;40:1230–4.
93. Morgan SL, Baggott JE, Vaughn WH, Young PK, Austin JV, Krumdieck CL, et al. The effect of folic acid supplementation on the toxicity of low-dose methotrexate in patients with rheumatoid arthritis. Arthritis Rheum 1990;33:9–18.
94. Morgan SL, Baggott JE, Vaughn WH, Austin JS, Veitch TA, Lee JY, et al. Supplementation with folinic acid during methotrexate therapy for rheumatoid arthritis: a double-blind, placebo-controlled trial. Ann Intern Med 1994;121:833–41.
95. Cutolo M, Sulli A, Pizzorni C, Seriolo B, Straub RH. Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis. Ann Rheum Dis 2001;60:729–35.
96. Cronstein BN. Molecular therapeutics: methotrexate and its mechanism of action. Arthritis Rheum 1996;39:1951–60.