Understanding the Mechanisms of Action of Methotrexate

Implications for the Treatment of Rheumatoid Arthritis

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Abstract

Methotrexate has been widely used for the treatment of rheumatoid arthritis (RA). The mechanisms of action of methotrexate are complex. Developed as a folic acid analogue, methotrexate inhibits purine and pyrimidine synthesis, which accounts for its efficacy in the therapy of cancer as well as for some of its toxicities. Recently, many studies have focused on the adenosine-mediated antiinflammatory effects of methotrexate. Certain aspects of methotrexate toxicities are also attributed to adenosine release. A better understanding of the mechanisms of action and toxicities of methotrexate will direct clinicians in their treatment approach and toxicity monitoring. Toward that objective, the latest developments in the pharmacokinetics, mechanism of action, pharmacogenetics, and toxicity of methotrexate are herein discussed.

Low-dose methotrexate was first demonstrated to be a potent and effective therapy for rheumatoid arthritis (RA) in 1985.[1] Because of its efficacy, acceptable safety profile, and low cost, methotrexate soon became a mainstay in the treatment of RA. More recently, new agents, including biological agents, have been compared to methotrexate for their efficacy during development. When begun earlier in the disease course, methotrexate is nearly as effective as biological response modifiers for the treatment of rheumatoid arthritis, although long-term follow-up suggests better prevention of bone destruction with biological agents. Methotrexate is now commonly administered in combination with either biological agents or other small-molecule antirheumatic drugs. Combination therapies have been reported to have greater efficacy than any single agent alone without greater toxicity.[2]

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Pharmacokinetics of Methotrexate

Methotrexate is generally administered once weekly to RA patients, with doses ranging from 7.5 to 25 mg/week.[3] It is well absorbed when given orally or intramuscularly. Intramuscular administration may help reduce side effects, especially nausea, which is commonly associated with oral ingestion. At the doses typically used for the treatment of RA, the bioavailability of oral methotrexate varies considerably between individuals, but generally it is approximately 70%. Oral absorption of methotrexate is not reduced by concomitant food intake. When taken orally, the uptake of methotrexate by the gastrointestinal tract is primarily mediated by a saturable transporter, reduced folate carrier 1 (RFC1). Therefore, oral bioavailability declines at higher doses when RFC1 is saturated. Parental administration of methotrexate may improve bioavailability by bypassing gastrointestinal transport. For example, absorption of methotrexate administered orally at 7.5 mg/week is roughly equivalent to that of a parentally administered drug, but absorption of oral methotrexate drops off by as much as 30% when the weekly dose is 15 mg or greater.

After absorption, 10% of methotrexate is converted to 7-hydroxymethotrexate in the liver. Both methotrexate and 7-hydroxymethotrexate are primarily excreted by the kidneys although a small portion is also excreted in the bile. Methotrexate is a drug with low-to-medium protein binding and high tissue distribution. It accumulates in the extravas-

Tian H, Cronstein BN. Understanding the mechanisms of action of methotrexate: implications for the treatment of rheumatoid arthritis. Bull NYU Hosp Jt Dis. 2007;65(3):168-73

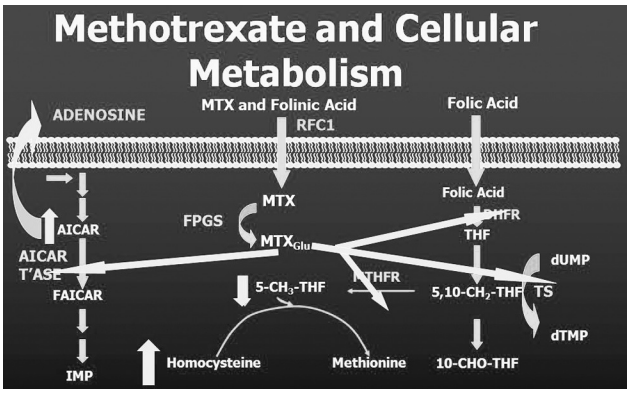

Figure 1 Methotrexate and cellular mechanism. 5-CH_3-THF indicates 5-methyl-tetrahydrofolate; 5,10-CH_2-THF, 5,10-methylene tetrahydrofolate; 10-CH0-THF, 10-formyl-tetrahydrofolate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyutodine monophosphate; FAICAR, formyl AICAR; FPGS, folypolyglutamate synthase; IMP, inosine monophosphate; MTHFR, methylene tetrahydrofolate reductase; MTX, methotrexate; MTX_{Glu}, methotrexate polyglutamate; RFC1, reduced folate carrier 1; T'ASE, transformylase; THF, tetrahydrofolate; and TS, thymidylate synthase.

cular pool, and dialysis only results in a transient decrease in drug concentration. The half-life of methotrexate in the serum is in the range of 6 to 8 hours after administration of the drug, and methotrexate is undetectable in the serum by 24 hours. Once taken-up by cells, a portion of methotrexate and 7-hydroxymethotrexate is metabolized to polyglutamate derivatives. Methotrexate polyglutamates (MTX_{Glu}) are stored in the tissues, including liver and erythrocytes, for long periods. The concentration of MTX_{Glu} in erythrocytes roughly correlates with the therapeutic efficacy of the drug.[4]

Methotrexate, as a folic acid antagonist, blocks the synthesis of purines and pyrimidines by inhibiting several key enzymes (Fig. 1). Inhibition of dihydrofolate reductase (DHFR) decreases tetrahydrofolate (THF) levels, which results in attenuated DNA/protein/lipid methylation, inhibition of thymidylate synthase (TS) interference with DNA synthesis, and inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase blocks de novo purine synthesis. The effect on purine and pyrimidine biosynthesis is also responsible for many toxicities of methotrexate, including bone marrow suppression, liver toxicity, and stomatitis. During the last 20 years, it has been documented that folic acid (1 to 5 mg/day) supplementation helps to prevent methotrexate associated toxicities.[2] Concomitant use of either folic acid or folinic acid (high doses of folinic acid being an exception) with methotrexate has no impact on the therapeutic efficacy of methotrexate in multiple clinical trials and meta-analyses. The latest ACR (American College of Rheumatology) guidelines, published in 2002, suggest that folic acid or folinic acid may be useful in the prevention of complications of methotrexate therapy. Inhibition of the antiinflammatory effects of methotrexate by high-dose folinic acid likely results from decreased methotrexate uptake, since folinic acid and methotrexate compete for the same transporter (RFC1) for absorption from the gastrointestinal tract and for cellular uptake. Patients are advised to take folinic acid at least 12 hours after their methotrexate dose to avoid diminishing gastrointestinal and cellular methotrexate uptake.

Mechanisms of Methotrexate Action

Currently, several pharmacological mechanisms of methotrexate action have been suggested, including inhibition of purine and pyrimidine synthesis, suppression of transmethylation reactions with accumulation of polyamines, reduction of antigen-dependent T-cell proliferation, and promotion of adenosine release with adenosine-mediated suppression of inflammation.[2] It is possible that a combination of these mechanisms is responsible for the antiinflammatory effects of methotrexate. To date, the adenosine-mediated antiinflammatory effect of methotrexate is best supported by the in vitro, in vivo, and clinical data.

Methotrexate Increases Extracellular Adenosine Release

The pathway leading to increased extracellular adenosine by methotrexate has been mostly delineated and supported by both in vitro and in vivo evidence.[2] Methotrexate and its metabolite, 7-hydroxymethotrexate, are first taken up by cells, where they undergo polyglutamation. MTX_{Glu} has been shown to be even more active than methotrexate as an inhibitor of folate-dependent enzymes (see above). AICAR transformylase inhibition by MTX_{Glu} leads to intracellular

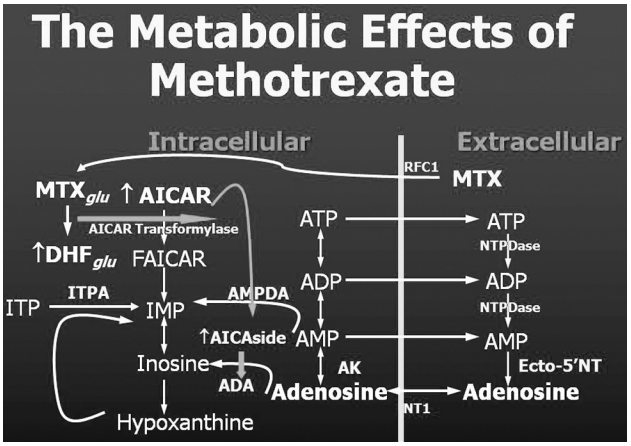

Figure 2 Methotrexate increases extracellular adenosine concentrations. MTX, methotrexate; MTX_{Glu}, methotrexate polyglutamate; DHF_{Glu}, dihydrofolate polyglutamate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; FAICAR, formyl AICAR; AMPDA, AMP deaminase; AICAside, aminoimidazole carboxamidoribonucleoside; ADA, adenosine deaminase; AK, adenosine kinase; RFC1, reduced folate carrier 1; NTPDase, nucleoside triphosphate dephosphorylase; Ecto-5'NT, ecto-5'-nucleotidase; NT1, nucleoside transporter 1.

accumulation of AICAR. Because AICAR inhibits AMP deaminase (AMPDA), an increased AICAR level leads to the release of intracellular adenine nucleotides and adenosine, either or both, into the extracellular space. Extracellular AMP, ADP, and ATP can be dephosphorylated to adenosine by the serial actions of ecto-apyrase (CD39) and ecto-5'-nucleotidase (Ecto-5'NT or CD73) (Fig. 2).

There is clear evidence that methotrexate treatment leads to AICAR accumulation.[4] Consistent with the observation that MTX_{Glu} is a potent inhibitor of AICAR transformylase, initial studies showed increased excretion of aminoimidazole carboxamide (AICA), a metabolite of AICAR, in urine of leukemia patients taking large doses of methotrexate. Further studies confirmed that low-dose MTX therapy increases tissue AICAR levels in animal models of RA and urinary AICA levels in patients with psoriasis and RA. Our in vivo data confirmed that pharmacologically relevant doses of methotrexate induce intracellular AICAR accumulation in splenocytes and increase adenosine concentrations in inflammatory exudates.[5]

Studies by Morabito and colleagues first determined whether methotrexate induced an increase of extracellular adenosine via intracellular adenosine production or was generated extracellularly from adenine nucleotides.[6] They demonstrated that injection of a specific ecto-5'NT inhibitor, α,β-methylene ADP, suppressed the antiinflammatory properties of methotrexate in a murine air pouch model of inflammation, indicating that adenosine released from methotrexate treatment is derived from adenine nucleotides converted extracellularly to adenosine. Recent investigations by Montesinos and coworkers further confirmed this hypothesis in Ecto-5'NT knockout mice, using the same air pouch model.[7] Treatment of wild-type mice with methotrexate increased adenosine concentrations and inhibited TNFα accumulation in inflammatory exudates. In contrast, methotrexate treatment did not affect adenosine levels and had no effect on TNFα accumulation in exudates of Ecto-5'NT knockout mice.

Adenosine has a very short half-life of 2 to 8 seconds in peripheral blood and body fluids. It is extremely difficult to measure the changes in adenosine levels in biological fluids, and, in most cases, improper sample collection renders the measurements uninterpretable. It is not surprising that conflicting data have been reported on adenosine levels in blood and inflammatory exudate in patients on methotrexate treatment. Recently, Riksen and associates demonstrated altered adenosine kinetics in RA patients on methotrexate treatment.[8] They took advantage of the potent vasodilating effects of adenosine and examined forearm blood flow (FBF) responses to adenosine before and after methotrexate treatment as a surrogate marker for methotrexate-mediated alterations in adenosine release. These investigators observed that adenosine increased FBF much more following methotrexate treatment. Moreover, when they infused dipyridamole (an adenosine uptake inhibitor) into patients, they found a clear increase in FBF. This provides functional evidence that methotrexate therapy alters extracellular adenosine levels.

Antiinflammatory Effect of Methotrexate is Mediated by Adenosine

The evidence for an adenosine-mediated antiinflammatory action of methotrexate only became clear in the early 1990s. Initial studies demonstrated that methotrexate treatment of cultured endothelial cells and fibroblasts increased adenosine release.[9] The released adenosine diminished stimulated neutrophil adhesion in vitro. Subsequent in vivo studies confirmed that by increasing intracellular AICAR accumulation, methotrexate increased adenosine concentration and diminished leukocyte accumulation in inflammatory exudates in the murine air pouch model.[5]

Adenosine is an endogenous purine nucleoside that, following its release into the extracellular space, binds to specific adenosine receptors expressed on the cell surface. Adenosine is an important signaling molecule modulating a vast array of physiological functions. Adenosine receptors belong to the family of G protein-coupled 7-transmembrane-spanning receptors (Fig. 3). Four distinct subtypes are known, termed A_1, A_{2A}, A_{2B}, and A_3. The contribution of the

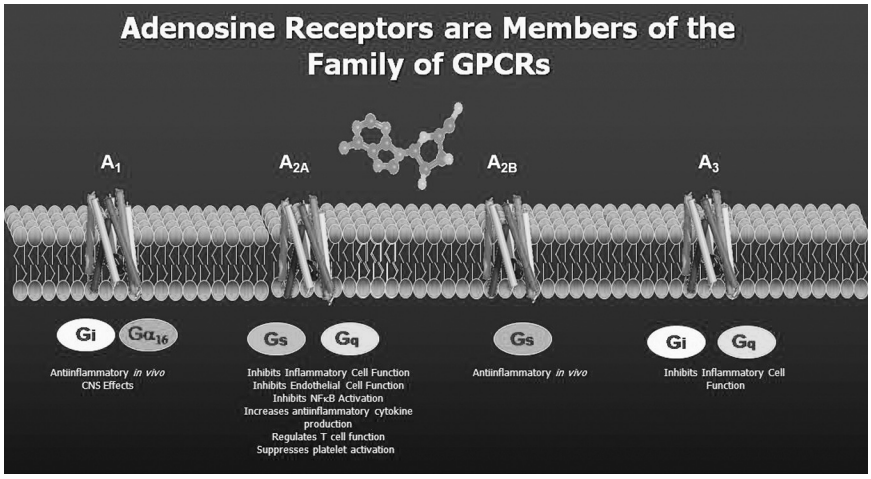

Figure 3 Adenosine receptors are members of the family of G-protein-coupled receptors.

specific adenosine receptor subtypes in various cells, tissues, and organs is complex, and the mechanisms involved in the endogenous regulation of inflammation by adenosine are still not currently understood.[10] However, extensive evidence supports the hypothesis that adenosine, acting on adenosine receptors, is a key mediator of the antiinflammatory effect of methotrexate.

The first evidence to establish that in vivo involvement of adenosine receptors in the mechanism of action of methotrexate is from the same murine air pouch model.[5] Methotrexate-mediated reduction in leukocyte accumulation was completely reversed by a specific adenosine A_{2A} receptor antagonist, but not affected by an adenosine A_1 receptor antagonist. Recent studies using adenosine receptor knockout mice provide further corroborative evidence in support of the hypothesis that adenosine, acting at A_{2A} and possibly A_3 receptors, mediates the suppressive effects of methotrexate on acute inflammation.[11] An animal model of RA was used to study the effects of methotrexate on chronic inflammation.[12] Nonselective adenosine receptor antagonists, theophylline and caffeine, reversed the therapeutic effects of methotrexate on adjuvant arthritis of mice. Consistent with animal studies, coffee drinking has been associated with methotrexate treatment failure. In a cohort of 91 RA patients, 26% of regular coffee drinkers discontinued methotrexate treatment, while the discontinuation rate among minimal coffee drinkers was 2%. Eighty percent of regular coffee drinkers who discontinued methotrexate did so due to lack of efficacy.[13] In a subsequent prospective study of 39 RA patients, Nesher and colleagues found that coffee could reverse some benefits of methotrexate treatment in patients with RA.[14] However, a recent retrospective study of 264 RA patients on methotrexate treatment for at least 1 year did not reveal a statistically significant attenuation of methotrexate efficacy among coffee drinkers.[15] The investigators argued that one explanation could be that adenosine receptor desensitization from chronic stimulation occurs among their patient population who have been on methotrexate treatment for a long time. This study is also limited by the fact that patients who discontinued methotrexate before 1 year of therapy, for the most part, were excluded from this analysis. A large control trial is needed to investigate the exact effects of caffeine on methotrexate efficacy in RA patients.

Pharmacogenetics of Methotrexate in the Treatment of RA

In recent years, researchers have been trying to determine genetic factors that modulate responses to certain drugs for rheumatic diseases. Investigations of single-nucleotide polymorphisms (SNPs) in genes encoding proteins critical in pharmacokinetic pathways could generate important information regarding the toxicity of and therapeutic response to certain drugs. Specific to methotrexate, SNPs in methylene tetrahydrofolate reductase (MTHFR) are consistently associated with enhanced methotrexate-mediated bone marrow toxicity but only poorly correlated, at best, with response to methotrexate therapy.[16] The genetic aspects of methotrexate efficacy have been shown to be multifactorial, which is not surprising since the antiinflammatory effects of methotrexate are complex. For example, Dervieux and coworkers suggested that combinations of functionally significant SNPs in RFC1, AICAR transformylase, and TS are more predictive of methotrexate response than any single SNP.[17]

Wessels and associates first investigated the relationship between SNPs in five genes involving the adenosine pathway and the outcome of methotrexate treatment.[18] In this subcohort analysis of 247 patients with recent-onset RA, they found that three SNPs in three genes were associated with good clinical response, and the greatest benefit was seen in patients with SNPs in all three genes. The same group then expanded their studies to determine 17 SNPs in 13 different genes related to the purine and pyrimidine synthesis among 205 newly diagnosed RA patients.[19] They proposed a scoring system to predict the efficacy of methotrexate as initial treatment for RA based on combined clinical and genetic factors. Although it is far from being practical, this pharmacogenetic model sheds light on individualized treatment of rheumatic diseases.

Methotrexate Toxicity

Patients taking methotrexate are more likely to discontinue therapies because of the adverse effects of medication rather than lack of efficacy. However, compared with other DMARDs (disease modifying antirheumatic drugs), including biological agents, methotrexate has a relatively good safety profile. The adverse effects of low-dose methotrexate are usually mild, self-limited, or preventable, but may be more severe in some patients. Toxicities from folate antagonism, including anemia, neutropenia, stomatitis, and oral ulcers, can be prevented or alleviated by folate supplementation. Toxicities unrelated to suppression of folate metabolism include nodulosis, hepatic fibrosis, pulmonary fibrosis, lethargy, fatigue, and renal insufficiency. Since adenosine, acting on adenosine receptors, plays an important role in antiinflammatory effects of methotrexate, many studies have investigated the role of the adenosine pathway in methotrexate toxicities. Information from these studies is important in new drug design since selective modulation of the adenosine pathway can maintain the efficacy while diminishing toxicity of the drug.

Merrill and colleagues studied methotrexate-induced nodulosis using an in vitro giant cell formation model.[20] They demonstrated that treatment of purified monocytes with methotrexate leads to enhanced cellular fusion and formation of multiple nucleated giant cells by phorbol ester. This effect of methotrexate is mediated by adenosine through the adenosine A_1 receptor since giant cell formation is reversed in vitro by a specific adenosine A_1 receptor antagonist. Methotrexate-induced hepatic fibrosis was recently found to be mediated through an adenosine pathway as well.[21]

Methotrexate, as well as ethanol, increases adenosine release from cultured hepatoma cells. Treatment of mice with a hepatotoxin leads to increased adenosine release from liver slices cultured ex vivo. Unlike wild-type mice, adenosine A_{2A} receptor knockout mice were protected from developing liver fibrosis in response to hepatotoxin. Moreover, an adenosine A_{2A} receptor-selective antagonist and nonselective adenosine receptor antagonist, caffeine, prevent hepatic fibrosis in mice treated with this same hepatotoxin. These results demonstrate that adenosine A_{2A} receptors play an important role in the pathogenesis of hepatic fibrosis, consistent with previous observations that adenosine A_{2A} receptors promote tissue repair, wound healing, and matrix production.[22] Recent studies also demonstrated the active role of adenosine A_{2A} receptors in diffuse dermal fibrosis.[23]

Conclusion

Our knowledge on the mechanism of methotrexate treatment for RA and methotrexate toxicity has been expanding in recent years although more animal and patient studies are needed. Research on the molecular biology and pharmacology aspects of such medications as methotrexate broadens our understanding of the pathogenesis of rheumatic diseases as well. Even in the era of biological agents, current treatment of RA is far from satisfactory in some patients. Our understanding of the pathogenesis of disease itself and the mechanism of antirheumatic drugs will form the basis for the development of new and more effective therapy.

Disclosure Statements

Dr. Tian has no financial interest or other activities to report associated with the article topic. Dr. Cronstein has patents on use of adenosine A_{2A} receptor agonists to promote wound healing and use of A_{2A} receptor antagonists to inhibit fibrosis, a patent on testing for SNPs in the adenosine A_1 receptor in patients with fibromyalgia, and a patent on use of adenosine A_1 receptor antagonists to treat osteoporosis and other diseases of bone. He has been a consultant within the past 2 years for Amgen, Bristol-Myers Squibb, CanFite Biopharmaceuticals, Cellzome, Endocyte, King Pharmaceutical (licensee of patents above), Prometheus Laboratories, Protalex, Regeneron (Westat, DSMB), Sepracor, and Tap Pharmaceuticals. He has received honoraria for the Speakers' Bureaus for Amgen and Tap Pharmaceuticals. Dr. Cronstein owns stock in CanFite Biopharmaceuticals (received for membership on the Scientific Advisory Board). He has also received research grants from King Pharmaceuticals and the NIH.

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