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Methotrexate and Erythro-9-(2-hydroxynon-3-yl) Adenine Therapy for Rat Adjuvant Arthritis and the Effect of Methotrexate on *In Vivo* Purine Metabolism

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Abstract

The objectives were: 1) to test the association of methotrexate (MTX) efficacy in rat adjuvant arthritis (rat AA) with interference of purine biosynthesis and adenosine metabolism and 2) to test the efficacy of erythro-9-(2-hydroxynon-3-yl) adenine (EHNA), an inhibitor of adenosine deaminase, and the efficacy of aminoimidazolecarboxamide (AICA) riboside plus MTX in rat AA. Radiographic and histologic examinations of the hind limbs were measures of efficacy. Urinary excretions of AICA and adenosine were markers of AICA ribotide transformylase inhibition (ie, blockage of purine biosynthesis) and interference with adenosine metabolism, respectively. AICA and adenosine excretions increased during the day of MTX dosing (treatment day) compared to the previous baseline day in animals responding well to MTX (ie. low radiographic and histologic scores). Based on radiographic and histologic scores, adjuvant injected rats were separated into two disease categories (ie, no/mild and moderate/severe). Only AICA excretion was significantly elevated on the treatment day in rat AA with no/mild disease (ie. those responding well to MTX therapy). AICA (not adenosine) excretion was significantly correlated with the above scores. EHNA was not efficacious, even at toxic levels, while AICA riboside potentiated the efficacy of MTX. The data suggests that efficacious MTX therapy in rat AA 1) blocks purine biosynthesis; 2) increases in *in vivo* AICA levels. Also adenosine accumulation and blockage of adenosine deaminase (i.e., by EHNA) appear to be less critical to MTX efficacy. Increased levels of AICA metabolites may suppress the immune response in rat AA

Keywords

Rat adjuvant arthritis; Methotrexate; Purine metabolism; Adenosine; Aminoimidazolecarboxamide; Erythro-9-(2-hydroxynon-3-yl) Adenine

Introduction

Low-dose methotrexate (MTX) is efficacious in the treatment of rheumatoid arthritis (RA); yet, the molecular mechanism(s) remains to be established. MTX is a disease modifying anti arthritic drug. We postulated that MTX efficacy is produced by the inhibition of purine

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nucleotide biosynthesis at the step catalyzed by aminoimidazolecarboxamide (AICA) ribotide transformylase (AICAR T'ase) plus the interference with adenosine metabolism, resulting from elevated AICA levels (Figure 1). We postulated that inhibition of adenosine deaminase would produce efficacy and that increasing AICA or metholites would potentiate MTX efficacy. These hypotheses were tested using the rat adjuvant arthritis (rat AA) model which resembles RA (Pearson, 1956: Morgan et al., 2001). Urinary AICA and adenosine excretions were measured to evaluate the AICAR T'ase (and purine nucleotide biosynthesis) inhibition and interference with adenosine metabolism, respectively (Figure 1). Increases in urinary adenosine and/or AICA have been reported in rat AA and psoriatic patients receiving MTX (Baggott et al., 1998;Baggott et al 1999). However, there is no data using a large range of MTX doses that associates drug efficacy with either AICA or adenosine levels. The efficacy of erythro-9-(2-hydroxynon-3-yl) adenine (EHNA), a potent inhibitor of adenosine deaminase (Schaeffer and Schwender, 1974;Agarwal et al, 1977), and the efficacy of MTX plus AICA-riboside were also tested in rat AA.

Methods

Animals

The rat AA model and the protocol used for MTX dosing have been described previously (Morgan et al., 2001;Baggott et al., 1998). This study was reviewed and approved by the Animal Use Committee of the University of Alabama at Birmingham. In the first set of experiments, five days after the injection of adjuvant at the base of the tail, intraperitoneal injections of 0 (solvent alone), 0.3, 0.5, 1, 2 and 3 mg of MTX/kg of body weight/week were given in two equal doses (ie. Tuesdays and Fridays). The animals receiving these doses are designated as the 0-, 0.3-, 0.5-, 1-, 2- and 3-MTX groups, respectively. A second set of experiments were performed using 0.5 mg MTX plus 20 mg AICA riboside/kg of body weight/week and 1 and 3 mg EHNA/kg of body weight/week. Animals receiving these doses (also on Tuesdays and Fridays) are designated 0.5-MTX+AICA, 1-EHNA and 3-EHNA, groups respectively. MTX, AICA-riboside and EHNA (Sigma Chemicals) were dissolved in phosphate-buffered saline (PBS). All animals received AIN 93M diet and water *ad libitum* in a light-cycle- and humidity-controlled room (Morgan et al. 2001). The 2- and 3-MTX groups were combined to yield a larger number of animals per group, since both of these doses resulted in premature death (Morgan et al. 2001). A group not injected with adjuvant but receiving PBS injections alone served as controls. In the first set of experiments at the end of the fourth-week of MTX treatment, a 24-hr urine was collected on the day before the MTX dose ("baseline day") and the day immediately following the MTX dose ("treatment day"). Additional 24 hour urines were collected on non-adjuvant injected animals.

At the end of the 6 week of treatment in both sets of experiments final body weights were measured and the animals were killed for histologic (first set of experiments) and radiologic (first and second sets of experiment) examinations of the hind limbs.

Biochemical, radiographic, histologic and statistical analyses

Urinary AICA, adenosine and creatinine assays were performed as previously described with the exception that AICA extractions were scaled down to accommodate a 5mL urine sample (Baggott et al., 1998;Baggott et al. 1999). Radiographic and histologic procedures and their scoring were also performed as previously described. (Morgan et al., 2001;Baggott et al., 1998). Histologic examination involved only right hind limbs, whereas both hind limbs were subjected to radiographic analysis. Mean radiographic scores, histologic scores, changes in body weights, and non-paired urinary AICA and adenosine levels were compared using the Williams test (Shirley, 1977). The paired *t*-test was used to evaluate the differences in urinary AICA and adenosine excretions using paired baseline day and treatment day data. Mean

radiographic and histologic scores were correlated with mean urinary AICA and adenosine excretion using linear regression. A $p<0.05$ was considered significant.

Results

Radiographic and histologic scores and body weight changes in MTX treated animals

Table 1 shows that there were large variations in the radiographic and histologic scores within the groups. For example, the radiographic scores for the 0- and 0.3-MTX groups ranged from 1 (mild) to 33 or 41 (severe), and the histologic scores ranged from 0 (no disease) to 11 or 12 (severe). However, the animals in the 1- and 2+3-MTX groups had significantly lower mean radiographic and histologic scores than the 0-MTX group, indicating that MTX had efficacy at these dose levels. There was also a MTX dose dependency in lowering mean radiographic scores. Maximum mean weight gain of 78 grams was observed in the 1-MTX group and was similar to that (i.e., 76 grams) observed in control animals (n=8) not receiving adjuvant.

Urinary AICA and adenosine in MTX treated animals

It is possible that adjuvant arthritis itself would have an effect on urinary AICA and adenosine excretion. It is also possible that remaining in a metabolic cage for 2 days produces stress that again alters urinary excretion of AICA and adenosine. Table 2 shows the mean AICA and adenosine excretion on the baseline day and the treatment day for the 0-MTX group and for controls which were not injected with adjuvant. No significant differences in the mean AICA or adenosine excretion were detected with disease activity of untreated animals or days in metabolic cage as the variables (Table 2).

Table 3 shows the mean adenosine excretion on baseline day and the day of MTX treatment. Only, the highest MTX dose (i.e. 2+3-MTX group) produced an increase in adenosine excretion above the O-MTX group during the treatment day. This group also increased adenosine excretions from baseline to treatment day. Table 4 shows that mean AICA excretion increased on treatment day from baseline day only in the 1-MTX group. However, baseline day AICA excretion was higher in the 0.3-MTX and 2 + 3-MTX groups when compared to the 0-MTX group. All groups treated with MTX had higher AICA excretion on the treatment day when compared to the 0-MTX group. Thus comparing treatment day level, there was a stronger MTX dose dependency in increasing urinary AICA excretion than in increasing urinary adenosine excretion.

Association between biochemical data and radiographic and histologic scores in MTX treated animals

We evaluated whether the large variation in disease activity (Table 1) was associated with a variation in AICA and adenosine excretions. Animals, independent of the MTX dose, which received adjuvant injections, were divided into two groups based on radiographic and histologic scores. Animals with no or mild disease activity had radiographic scores of 0 to 2.5 and histologic scores of 0 to 1.5. Animals with moderate or severe disease activity had radiographic scores of 5.5 to 41 and histologic scores of 4 to 12. Thus, this type of analysis eliminated those animals with disease activities between these extremes. The animals with no or mild disease activity are responding well to MTX therapy, and animals with moderate and severe disease activity are primarily in the 0-MTX group plus those animals with a poor response to MTX therapy. Table 5 shows mean AICA and adenosine excretion of animals categorized into the no/mild and moderate/severe disease activities. Mean AICA excretion was significantly higher during the MTX treatment day in animals whose disease activity was low, and therefore, responding well to MTX therapy when compared to animals in the 0-MTX group and animals who responded poorly to MTX therapy (ie. moderate/severe disease activity). In contrast, mean adenosine excretion was not different in this type of analysis.

Higher mean treatment day urinary AICA excretion (from table 4) were significantly correlated with lower mean radiographic and histologic scores (from table 1) with r^2 of 0.96 and 0.99 ($p < 0.05$), respectively. In contrast mean treatment day urinary adenosine excretion (from table 3) were not significantly correlated with the above scores, $r^2 = 0.32$ and 0.48 ($p > 0.05$), respectively.

Effect of EHNA and AICA-riboside plus MTX treatment on rat AA

The potent adenosine deaminase inhibitor, EHNA, was given in order to test whether direct interference with adenosine metabolism would be efficacious in rat AA. As shown in Table 6, neither dose of EHNA was efficacious compared to the 0-MTX group, nor was there any suggestion of a dose dependency. The 3-EHNA dose level resulted in premature death of two of the eight animals.

AICA-riboside was given along with a suboptimal dose of MTX (i.e., 0.5-MTX) in order to test whether the former could potentiate the efficacy of the latter in rat AA. Both the 0.5-MTX and 0.5-MTX+AICA doses resulted in mean radiographic scores significantly lower than the 0-MTX group with significantly higher body weight changes (Table 6). The mean radiographic score of the 0.5-MTX + AICA group was lower than all other groups, suggesting that AICA-riboside potentiated MTX efficacy. Mean body weight changes in the 0.5-MTX and 0.5-MTX + AICA groups were similar to that (i.e., 62 grams) observed for controls (n=8) not receiving adjuvant.

Discussion

Interference with adenosine metabolism is a hypothesis (“adenosine hypothesis”) possibly explaining the efficacy of low-dose MTX therapy for autoimmune disease (Baggott et al., 1999;Cronstein et al., 1994;Baggott et al., 1993;Cronstein et al., 1993;Gadangi et al., 1996). MTX treatment produces increased *in vivo* levels of adenosine and/or its metabolites, and this leads to the suppression of the immune system. Some of our data are in agreement with this hypothesis. For example, MTX therapy in the 2+3-MTX group showed increased urinary adenosine excretion and efficacy (Table 3). However, when the data were analyzed with disease activities as variables, no significant association between adenosine excretion and MTX efficacy was found (Table 5; $r^2 = 0.32, 0.48$). A note of caution is in order; we did not measure hour-by-hour changes in urinary adenosine or AICA excretion following the MTX doses. Therefore, we might have missed substantial elevations of these metabolites within a few hours of the dose. Also, adenosine or AICA found in urine may not parallel intracellular concentrations of these metabolites. However, in an additional set of experiments, direct inhibition of adenosine metabolism was tested. EHNA is a semi-tight-binding inhibitor of adenosine deaminase with a Ki of 1 to 6×10^{-9} M and should therefore increase *in vivo* adenosine levels (Schaeffer, Schwender, 1974;Agarwal et al., 1977). However, EHNA, even at toxic levels, was not efficacious, suggesting that blocking this adenosine metabolism is relatively unimportant in the mechanism of action of MTX in rat AA (Table 6).

The adenosine hypothesis is controversial for several reasons (Montesinos et al., 2000; Aderson et al., 2000). If this hypothesis is true, adenosine-receptor antagonists should reduce the efficacy of MTX. In rat AA, administration of two adenosine-receptor antagonists (ie. caffeine and theophylline) have been reported to markedly reduce the efficacy of MTX (Montesinos et al., 2000). In contrast, other adenosine-receptor antagonists have been reported to markedly increase the MTX efficacy in the rat antigen-induced arthritis (Anderson et al., 2000). A possible explanation for these contradictory findings is that caffeine and theophylline bind to all sub-types of adenosine receptors, whereas the other antagonists have different binding affinities for the various receptor subtypes. Binding of the antagonists to adenosine receptors can result in both anti-inflammatory and pro-inflammatory responses. For example, although

activation of A_{2A} receptors is anti-inflammatory, activation of A_1, A_{2B} and A_3 receptors are pro-inflammatory (Linden, 2001). Nesher, et al., (2003) reported that heavy caffeine consumption by RA patients significantly reduced some indices of MTX efficacy, which would be consistent with the animal data (Montesinos et al., 2000). In contrast, a larger study of caffeine consumption by RA patient there was no significant reduction in any index of MTX efficacy when comparing mean caffeine consumption of 422 mg/day to 39 mg/day (Benito-Garcia et al, 2006).

Our data does indicate that purine biosynthesis is inhibited by MTX at the AICAR T'ase step producing an *in vivo* accumulation and excretion of AICA (and most likely its metabolites) (Table 4). Higher AICA excretion was correlated with better MTX efficacy (Table 5; $r^2 = 0.96$, 0.99). Others have reported similar increases in AICA metabolites using different animal models (Baggott et al., 1999;Cronstein et al., 1994;Baggott et al., 1993;Cronstein et al., 1993;Gadangi et al., 1996). For example, both MTX and sulfasalazine produce about a three-fold increase in cellular AICA-ribotide (Cronstein et al., 1994;Cronstein et al., 1993;Gadangi et al., 1996). We previously reported increased 24-hr urinary AICA excretion in rat AA and psoriatic patients after MTX treatment (Baggott et al., 1998;Baggott et al., 1999). In a test of the efficacy of AICA (and metabolites), we found that addition of AICA riboside to a suboptimal MTX dose potentiated the effect of MTX (Table 6). Therefore, it may be reasonable to conclude that the efficacy of MTX could be partly mediated by accumulation of AICA and its metabolites and not solely mediated by an interference with adenosine metabolism. However AICA-riboside plus MTX may also have increased adenosine excretion.

In support of this idea, AICA and its metabolites appear to have biological effects independent of adenosine metabolism. The administration of AICA riboside to humans and animals has been reported to reduce the inflammation and oxidative injury of ischemia reperfusion (McGee et al., 1995;Hori et al., 1994;Galinanes et al., 1992;Clough-Helfman, Phillis, 1990). AICA ribotide activates AMP-activated protein kinase, and the down-stream effects of this stimulation inhibits metabolic pathways including glycolysis, fatty acid biosynthesis, cholesterol biosynthesis and gluconeogenesis (Ojuka et al., 2000; Russel et al., 1999; Merrill et al., 1997;Velasco et al., 1997;Henin et al., 1996). AICA–riboside inhibits cancer cell growth in both in vitro and in vivo experiments via activation of AMP – activated protein kinase (Rattan et.al, 2005). Global metabolic changes mediated by AICA ribotide activation of this kinase have been reviewed (Hardie and Hawley, 2001) and these changes may suppress immune function.

In addition to these global metabolic effects of AICA ribotide, specific effects on energy, carbohydrate and amino acid metabolism are known. AICA metabolites can waste energy in futile ATP dependent cycles (Vincent et al., 1996). In rat hepatocytes, AICA metabolites directly inhibit glucokinase and block the formation of fructose 2,6-bisphosphate, an important activator of 6-phosphofructo-1-kinase and the net effect is an inhibition of glycolysis (Vincent et al., 1992). The concentrations of AICA compounds used in these experiments ranged between 10 and 100 μM, which can be achieved in lymphoblasts exposed to only 20 nM MTX (Bokkerink et al., 1986; Bokkerink et al., 1988), or in inflammatory cells of animals treated with low-dose MTX (Cronstein et al., 1994;Cronstein et al., 1993;Gadangi et al., 1996). Glycolysis is an important energy-producing pathway for the immune system since leukocytes do not have an active pyruvate dehydrogenase complex; thus, energy derived from the Krebs cycle cannot be obtained using glucose as the metabolic fuel (Biswas et al., 1998;Haji-Michael, et al., 1999). In cell culture experiments, AICA riboside potentiated MTX toxicity in neoplastic T cells, an analogous finding to its potentiation of MTX efficacy in rat AA that we report here (Ha and Baggott, 1994). In other cancer cell cultures MTX potentiated AICA–riboside's toxicity (Becker et.al, 2006). AICA riboside also irreversibly inactivated S-adenosylhomocysteine hydrolase, an enzyme important for the use of S-adenosylmethionine

in methylation reactions (Ha and Baggott, 1994). Inhibitors of S-adenosylhomocysteine hydrolase are efficacious in the treatment of animal models of autoimmune disease (Fu et.al., 2006). Thus, AICA metabolites interfere with various metabolic pathways and processes other than those exclusive to adenosine metabolism, and this interference may suppress immune function.

It should be emphasized that MTX inhibits purine nucleotide biosynthesis de novo and that this may be immunosuppressive independent of which step is inhibited (Bokkerink, et al., 1986). MTX inhibition of purine nucleotide biosynthesis may be just as detrimental to the immune system as inhibition of this pathway by 6-mercaptopurine, a known immunosuppressive agent (Stet et al., 1993). Bypassing this block by giving purines, including hypoxanthine, inosine and even adenosine, reverses 6-mercaptopurine inhibition of the human mixed-lymphocyte response (Al-Safi and Maddocks, 1984). Smolenska *et al.* (1999) reported decreased plasma hypoxanthine and uric acid concentrations without an increase in plasma adenosine or erythrocyte AICA-ribose-triphosphate in patients with RA receiving their initial MTX dose. Their finding is consistent with simple inhibition of purine nucleotide biosynthesis as a mechanism contributing to MTX efficacy and suggests that inhibition by MTX at any metabolic step (including AICAR T'ase) in purine biosynthesis is immunosuppressive in and of itself.

In a study in RA patients receiving MTX, increased urinary excretion of AICA (but not adenosine) was correlated with MTX efficacy (Morgan et.al., 2004). Recently, Dolezalova et al. (2005) have reported that blood adenosine levels in juvenile arthritis patients were not significantly associated with or correlated with MTX therapy, MTX dose, erythrocyte MTX-polyglutamate levels or clinical response to MTX. From the above data and the data presented here, we conclude that the immunosuppressive effects of MTX are due in part to a block in purine nucleotide biosynthesis at the AICAR T'ase step in concert with global and specific metabolic effects (which may be immunosuppressive) of increased *in vivo* levels of AICA and its metabolites.

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References

Agarwal RP, Spector T, Parks RE Jr. Tight-binding inhibitors--IV. Inhibition of adenosine deaminases by various inhibitors. Biochem Pharm 1977;26:359–67. [PubMed: 849330]

Al-Safi SA, Maddocks JL. Azathioprine and 6-mercaptopurine (6-MP) suppress the human mixed lymphocyte reaction (MLR) by different mechanisms. Br J Clin Pharmacol 1984;17:417–22. [PubMed: 6232936]

Anderson SE, Johansson LH, Lexmuller K, Ekstrom GM. Anti-arthritic effect of methotrexate: is it really mediated by adenosine? Eur J Pharm Sci 2000;9:333–43. [PubMed: 10664473]

Baggott JE, Morgan SL, Ha TS, Alarcón GS, Koopman WJ, Krumdieck CL. Antifolates in rheumatoid arthritis: a hypothetical mechanism of action. Clin Exp Rheumatoid 1993;11 (Suppl 8):101–5.

Baggott JE, Morgan SL, Koopman WJ. The effect of methotrexate and 7-hydroxymethotrexate on rat adjuvant arthritis and on urinary aminoimidazole carboxamide excretion. Arthrit Rheum 1998;41:1407–10.

Baggott JE, Morgan SL, Sams WM, Linden J. Urinary adenosine and aminoimidazolecarboxamide excretion in methotrexate-treated patients with psoriasis. Arch Dermatol 1999;135:813–7. [PubMed: 10411156]

Beckers A, Organe S, Timmermans L, Vanderhoydonc F, Deboel L, Derua R. Methotrexate enhances the antianabolic and antiproliferative effects of 5-aminoimidazole- 4-carboxamide riboside. Mol Cancer Therapy 2006;5:2211–17.

Benito-Garcia E, Heller JE, Chibnik LB, Maher NE, Matthews HM, Bilics JA, et al. Dietary caffeine intake does not affect methotrexate efficacy in patiens with rheumatoid arthritis. J Rheumatol 2006;33:1275–81. [PubMed: 16821266]

Biswas S, Ray M, Misra S, Dutta DP, Ray S. Is absence of pyruvate dehydrogenase complex in mitochondria a possible explanation of significant aerobic glycolysis by normal human leukocytes? FEBS Letters 1998;425:422.

Bokkerink JP, Bakker MAH, Hulscher TW, De Abreu RAA, Schretlen EDAM. Purine de novo synthesis as the basis of synergism of methotrexate and 6-mercaptopurine in human malignant lymphoblasts of different lineages. Biochem Pharmacol 1988;37:2321–7. [PubMed: 2455519]

Bokkerink JPM, Bakker MAH, Hulscher TW, De Abreu RRA, Schretlen EDAM, vanLaarhoven JPRM, et al. Sequence-, time- and dose-dependent synergism of methotrexate and 6-mercaptopurine in malignant human T-lymphoblasts. Biochem Pharmacol 1986;35:3549–55. [PubMed: 2429667]

Clough-Helfman C, Phillis JW. 5-aminoimidazole-4-carboxamide riboside (AICAr) adminisstration reduces cerebral ischemic damage in the Mongolian gerbil. Brain Res Bull 1990;25:203–6. [PubMed: 2207710]

Cronstein BN, Naime D, Ostad E. The anti-inflammatory mechanism of methotrexate. Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an in vivo model of inflammation. J Clin Invest 1993;92:2675–82. [PubMed: 8254024]

Cronstein BN, Naime D, Ostad E. The anti-inflammatory effects of methotrexate are mediated by adenosine. Adv Exp Med Biol 1994;370:411–6. [PubMed: 7660940]

Dolezalova P, Krijt J, Chladek J, Nemcova D, Hoza J. Adenosine and methotrexate polyglutamate concentrations in patients with juvenile arthritis. Rheumatol 2005;44:74–9.

Fu Y-F, Zhu Y-N, Ni J, Zhong X-G, Tang W, Re Y-D, et al. A reversible S-adenosyl -L- homocysteine hydrolase inhibitor ameliorates experimental autoimmune encepholomyelitis by inhibiting T cell activation. J Pharm Exp Therap 2006;319:799–808.

Gadangi P, Logaker M, Naime D, Levin RI, Recht PA, Montesinos MC, et al. The anti-inflammatory mechanism of sulfasalazine is related to adenosine release at inflamed sites. J Immunol 1996;156:1937–47. [PubMed: 8596047]

Galinanes M, Bullough D, Mullane KM, Hearse DJ. Sustained protection by acadesine against ischemia- and reperfusion-induced injury. Studies in the transplanted rat heart. Circulation 1992;86:589–97. [PubMed: 1638724]

Ha T, Baggott JE. 5-aminoimidazole-4-carboxamide ribotide and its metabolites: metabolic and cytotoxic effects and accumulation during methotrexate treatment. J Nutr Biochem 1994;5:522–8.

Haji-Michael PG, Ladrière L, Sener A, Vincent JL, Malaisse WJ. Leukocyte glycolysis and lactate output in animal sepsis and ex vivo human blood. Metabolism 1999;48:779–85. [PubMed: 10381154]

Hardie DG, Hawley SA. AMP-activated protein kinase: The energy charge hypothesis revisited. BioEssays 2001;23:1112–9. [PubMed: 11746230]

Henin N, Vincent MF, Van den Berghe G. Stimulation of rat liver AMP-activated protein kinase by AMP analogues. Biochem Biophys Acta 1996;1290:197–203. [PubMed: 8645724]

Hori M, Kitakaze M, Takashima S, Morioka T, Sato H, Minamino T, et al. AICA riboside improves myocardial ischemia in coronary microembolization in dogs. Am J Physiol 1994;267:H1483–95. [PubMed: 7943395]

Leung JM, Stanley T III, Mathew J, Curling P, Barash P, Salmenpera M, et al. An initial multicenter, randomized controlled trial on the safety and efficacy of acadesine in patients undergoing coronary artery bypass graft surgery. Anesth Analgesia 1994;78:420–34.

Linden J. Molecular approach to adenosine receptors: Receptor mediated mechanisms of tissue protection. Ann Rev Pharmacol Toxicol 2001:775–87. [PubMed: 11264476]

McGee DS, Vinten-Johansen J, Van Wylen DG. Acadesine reduces myocardial infarct size by an adenosine mediated mechanism. Cardiovasc Res 1995;29:495–505. [PubMed: 7796443]

Merrill GF, Kurth EJ, Hardi DG, Winder WW. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am J Physiol 1997;273:E1107–12. [PubMed: 9435525]

Montesinos MC, Yap JS, Desai A, Posadas I, McCrary CT, Cronstein BN. Reversal of the anti-inflammatory effects of methotrexate by the nonselective adenosine receptor antagonists theophylline

and caffeine: evidence that the anti-inflammatory effects of methotrexate are mediated via multiple adenosine receptors in rat adjuvant arthritis. Arthrit Rheum 2000;43:656–63.

Morgan SL, Baggott JE, Bernreuter WK, Gay RE, Arani R, Alarcon GS. Methotrexate affects inflammation and tissue destruction differently in the rat adjuvant arthritis model. J Rheumatol 2001;28:1476–81. [PubMed: 11469449]

Morgan SL, Oster RA, Lee JY, Alarcon GS, Baggott SE. The effect of folic acid and folinic acid supplements on purine metabolism in methotrexate-treated rheumatoid arthritis. Arthrit Rheum 2004;50:3104–11.

Nesher G, Mates M, Zevin S. Effect of caffeine consumption on efficacy of methotrexate in rheumatoid arthritis. Arthrit Rheum 2003;48:571–2.

Ojuka EO, Nolte LA, Holloszy JO. Increased expression of GLUT-4 and hexokinase in rat epitrochlearis muscles exposed to AICAR in vitro. J Appl Physiol 2000;88:1072–5. [PubMed: 10710405]

Pearson DM. Development of arthritis, periarthritis and periositiitis in rats given adjuvants. Proc Soc Exp Biol 1956;91:95–101.

Rattan R, Giri S, Singh AK, Singh I. 5-aminoimidazole-4-carboxamide – 1 –β– D – ribofuranoside inhibits cancer cell proliferation *in vitro and in vivo* via AMP – activated protein kinase. J Biol Chem 2005;280:39582–93. [PubMed: 16176927]

Russell RR III, Bergeron R, Shulman GI, Young LH. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. Am J Physiol 1999;277:H643–9. [PubMed: 10444490]

Schaeffer HJ, Schwender CF. Enzyme inhibitors.26. Bridging hydrophobic and hydrophilic regions on adenosine deaminase with some 9-(2-hydroxy-3-alkyl) adenines. J Med Chem 1974;176:9.

Shirley E. A non-parametric equivalent of Williams' test for contrasting increasing dose levels of a treatment. Biometrics 1977;33:386–9. [PubMed: 884197]

Smolenska Z, Kaznowska Z, Zarowny D, Simmonds HA, Smolenski RT. Effect of methotrexate on blood purine and pyrimidine levels in patients with rheumatoid arthritis. Rheumatology 1999;38:997–1002. [PubMed: 10534552]

Stet EH, De Abreu RA, Bokkerink JPM, Vogels-Mentink TM, Lambooy LHJ, Trijbels FJM, et al. Reversal of 6-mercaptopurine and 6-methylmercaptopurine ribonucleoside cytotoxicity by aminoimidazole carboxamide ribonucleoside in Molt F4 human malignant T-lymphoblasts. Biochem Pharmacol 1993;46:547–50. [PubMed: 8347177]

Velasco G, Geelen MJ, Guzman M. Control of hepatic fatty acid oxidation by 5′-AMP-activated protein kinase involves a malonyl-CoA-dependent and a malonyl-CoA-independent mechanism. Arch Biochem Biophys 1997;337:169–75. [PubMed: 9016810]

Vincent MF, Bontemps F, Van den Berghe G. Inhibition of glycolysis by 5-amino-4-imidazolecarboxamide riboside in isolated rat hepatocytes. Biochem J 1992;281:267–72. [PubMed: 1531010]

Vincent MF, Bontemps F, Van den Berghe G. Substrate cycling between 5-amino-4-imidazolecarboxamide riboside and its monophosphate in isolated rat hepatocytes. Biochem Pharmacol 1996;52:999–1006. [PubMed: 8831718]

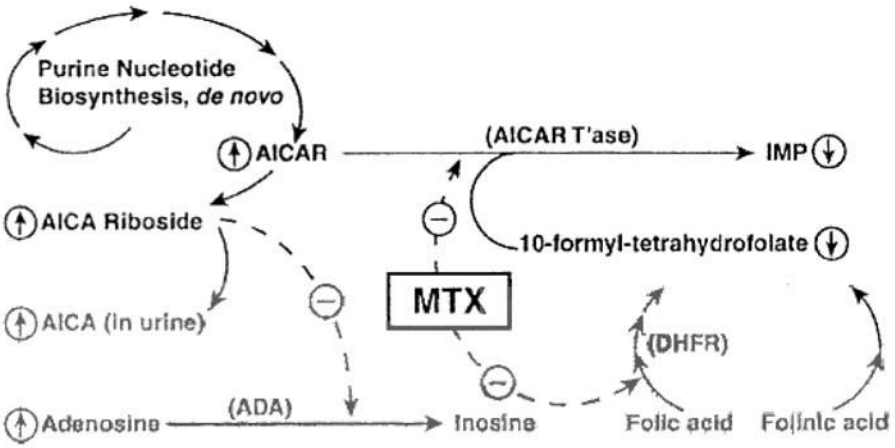

Figure 1.

Methotrexate (MTX) is shown inhibiting aminoimidazolecarboxamide (AICA) ribitide transformylase (AICAR T'ase) and dihydrofolate reductase (DHFR). *In vivo* levels of AICA riboside increase and inhibit adenosine deaminase (ADA). The urinary excretion of both AICA and adenosine increases.

Table 1

Mean Radiographic and Histologic Scores and Mean Body Weight Changes in MTX-Treated Rat AA

Group	N	Radiographic (range)[1]	Histologic (range)[2]	Body Weight Changes[3]
0-MTX	18	17.9 (1 – 41)	5.5 (0 – 12)	55(±3)
0.3-MTX	10	9.6 (1.5 – 33)	3.8 (0 – 11)	69(±4)
1-MTX	10	1.6 (0 – 3)	1.8 (0 – 4)	78(±2)
2+3-MTX	9	2.4 (1 – 4)	1.3 (0 – 4.5)	64(±5)

[1] The total radiographic score is the sum of the scores of the two hind limbs as described previously [3]. The maximum score for each limb was 28; therefore, the maximum score for both limbs is 56. The following means are significantly different: 1.6 < 17.9 and 9.6; 2.4 < 17.9.

[2] The total histologic score is the sum of the scores for the right hind limb as described previously [3]. The maximum score is 18. The following means are significantly different: 1.8 < 5.5; 1.3 < 5.5.

[3] Mean change (±SEM), in grams, from the beginning of the first week of MTX therapy to the end of the 6^{th} week of therapy. The following means are significantly different: 78 > 55 and 64; 69 > 55.

Table 2

The Effect of Adjuvant Arthritis and Stay in a Metabolic Cage on Mean 24-Hour Urinary AICA and Adenosine Excretion

Group	(n)	Adenosine[1] (mean ± SEM)	
		Baseline Day	Treatment Day
0-MTX	(18)	77 (±24)	68 (±22)
Controls	(8)	45 (±16)	65 (±26)
		AICA[1] (mean ± SEM)	
Group	(n)	Baseline Day	Treatment Day
0-MTX	(18)	82 (±16)	80 (±15)
Controls	(8)	139 (±13)	106 (±9)

[1] in μmoles/gram creatinine

Table 3

The Effect of MTX Dose on Baseline and Treatment Day Mean 24-Hour Urinary Excretion of Adenosine

Group	(n)	Adenosine[1] (mean ± SEM)	
		Baseline Day[2]	Treatment Day[2]
0-MTX	(18)	77 (±24)	68 (±22)
0.3-MTX	(10)	88 (±28)	91 (±19)
1-MTX	(10)	67 (±26)	87 (±24)
2 + 3-MTX	(9)	73 (±14)	240 (±57)

[1] in μmoles/gram creatinine

The following means are significantly different: 240 > 73, 68, 91 and 87.

Table 4

The Effect of MTX Dose on Baseline Day and Treatment Day Mean 24-Hour Urinary AICA Excretion

Group	(n)	AICA[1] (mean ± SEM)	
		Baseline Day[2]	Treatment Day[2]
0-MTX	(18)	82 (±16)	80 (±15)
0.3-MTX	(10)	156 (±30)	142 (±21)
1-MTX	(10)	101 (±19)	215 (±68)
2 + 3-MTX	(9)	142 (±28)	240 (±65)

[1] in μmoles/gram creatinine

The following means are significantly different 82 < 156 and 142 (baseline day); 80 < 142 (treatment day), 215 and 240; 101 < 215.

Table 5

Urinary Excretion of Adenosine and AICA Categorized by Disease Activity

Disease Activity[3]	(n)	Adenosine Excretion[1] (mean ± SEM)	
		Baseline Day[2]	Treatment Day[2]
Radiographic no/mild	(16)	94 (±24)	127 (±28)
Radiographic moderate/severe	(15)	77 (±26)	82 (±23)
Histologic no/mild	(18)	72 (±18)	104 (±25)
Histologic moderate/severe	(15)	77 (±27)	101 (±24)
		AICA Excretion[1] (mean ± SEM)	
Disease Activity[3]	(n)	Baseline Day[2]	Treatment Day[2]
Radiographic no/mild	(16)	127 (±20)	260 (±59)
Radiographic moderate/severe	(15)	83 (±18)	87 (±19)
Histologic no/mild	(18)	125(±18)	218(±53)
Histologic moderate/severe	(15)	93 (±22)	117 (±22)

[1] in micromoles/gram creatinine;

[2] for AICA excretion the following means are significantly different: 260 > 127 and 87; 218 > 125 and 117; no signicicant differences in means were found for adenosine excretion

[3] disease activity categories are described in the text

Table 6

Mean Radiographic Scores and Body Weight Changes in MTX, MTX plus AICA Riboside and EHNA treated Rat AA

Group	n	Radiographic (range)[1]	Body Weight Changes[2]
0-MTX	8	11.7 (4–27)	49 (±4)
0.5-MTX	8	4.6 (0–12)	66 (±5)
0.5-MTX + AICA	8	0.8 (0–2)	65 (±5)
1-EHNA	8	10.8 (5–32)	52 (±5)
3-EHNA	6	11.1 (0–43)	53 (±6)

[1] The total radiographic score is the sum of the scores of the two hind limbs as described previously [3]. The maximum score for each limb was 28; therefore, the maximum score for both limbs is 56. The following means are significantly different: 0.8 < 11.7, 11.1, 10.8 and 4.6; 4.6 < 11.7.

[2] Mean change (±SEM), in grams, from the beginning of the first week of MTX therapy to the end of the 6 th week of therapy. The following means are significantly different: 49 < 66 and 65.