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Review

# Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis

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This review presents recent data supporting the methotrexate (MTX) mechanisms of action, which are likely to account for its anti-proliferative and immunosuppressive effects in rheumatoid arthritis (RA). The effects of MTX *in vivo* may be mediated by reducing cell proliferation, increasing the rate of apoptosis of T cells, increasing endogenous adenosine release, altering the expression of cellular adhesion molecules, influencing production of cytokines, humoral responses and bone formation. Several reports indicate that the effects of MTX are influenced by genetic variants, specific dynamic processes and micro-environmental elements such as nucleotide deprivation or glutathione levels. The challenge for the future will be linking biological and genetic markers relevant to the response to MTX in RA.

**Key words:** Methotrexate, Molecular mechanisms of action, Pharmacology, Rheumatoid arthritis.



## Introduction

Methotrexate (MTX), the most frequently used disease-modifying anti-rheumatic drug (DMARD), suppresses disease activity and reduces joint damage [1]. The precise mechanism of action of folate antagonist MTX in the treatment of rheumatoid arthritis (RA) is unclear, although it is thought that MTX prevents *de novo* pyrimidine and purine syntheses, required for DNA and RNA syntheses, and consequently inhibits cellular proliferation of lymphocytes involved in the inflammation process.

At the cellular level, MTX and/or MTX-polyglutamates directly inhibit dihydrofolate reductase (DHFR), thymidylate synthase (TS) and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase. Other folate enzymes such as methylenetetrahydrofolate reductase (MTHFR) may be influenced indirectly (Fig. 1). MTX enters the cell via the reduced folate carrier (RFC1), whereas several multi-resistance proteins (MRPs) and P-glycoprotein (P-gp) probably facilitate cellular efflux [2, 3].

Despite the fact that it is known that MTX inhibits several enzymes, it is unclear how weekly low-dose MTX could account entirely for the immunosuppressive effects in RA. Indeed, suppression of disease activity, observed after a latent period of weeks and administration of 1–5 mg folic acid weekly to reduce MTX-induced adverse drug events, does not affect clinical efficacy [4]. Therefore, the biological and pharmacological effects of MTX are intensively studied and alternative mechanisms of actions are put forward to explain its clinical effects in RA [5, 6].

A better understanding of the mechanism of MTX action may be useful in identifying those RA patients who are most likely to benefit from treatment and thus may help optimizing therapy in RA. The molecular mechanism of action of low-dose MTX in RA has been reviewed by Cronstein and Cutolo *et al*. and includes literature data until 2004. These manuscripts demonstrate that MTX may act in RA through reducing cell proliferation, increasing the rate of apoptosis of T cells, increasing endogenous adenosine concentrations and altering cytokine production and humoral responses. Our article adds novel information following systematic literature retrieval including publications of the period January 2002 to 20 August 2007.

## Cell proliferation and apoptosis

Several reports show that the mechanism of MTX action on cell proliferation and apoptosis depends on the alteration of the intracellular reactive oxygen species (ROS) levels [7–12], on the inhibition of pyrimidine pathway enzymes [13], on increasing CD95 sensitivity of CD45 + RO cells (activated T cells) leading to increased apoptosis [14], on decreasing methyltransferase activity relevant for (de)activation of enzymes [15] and on the reduction of cellular micro-environment elements such as nucleotide or natural folates [16, 17].

Most studies describe a dose- and time-dependent effect of MTX on inhibition of cell proliferation and induction of apoptosis. [7, 9, 11, 13, 16]. One study shows that MTX induces apoptosis in activated leucocytes and resting T cells [14], although apoptosis in resting T cells could not be detected in other studies [7, 8, 12]. It suggests that MTX induces apoptosis in highly activated cells only.

Differences seem to exist between monocytes and lymphocytes with regard to intracellular ROS production levels, since MTX was found to be cytotoxic to a different extent for lymphocytic and monocytic cell lines after 24 h of incubation [9]. In three lymphocytic and two monocytic cell lines, ROS levels increased in a time-dependent manner with a maximum at 4 h of incubation. However, a correlation between apoptosis and ROS generation was shown in the lymphocytic cell lines only [9]. Others confirmed the time- and dose-dependent ROS-induced apoptosis through MTX in Jurkat T cells, without MTX inducing apoptosis in monocytes. As a consequence, the cytotoxic effect of MTX in monocytes is thought to be due to a different mechanism of action than ROS production. It was suggested that monocytes exhibit a higher intrinsic level of antioxidants, such as increased glutathione levels, which prevents ROS-induced apoptosis [7].

Indirect evidence for MTX mechanism of actions on cell proliferation and apoptosis through increasing ROS production is given by studying the role of ornithine decarboxylase (ODC) overexpression [11, 12]. ODC is the first and rate-limiting enzyme of the polyamine pathway, it decarboxylates L-ornithine to putrescine (Fig. 2). Overexpression of ODC leads to increased polyamine levels, spermine and spermidine, which are ROS scavengers. The proposed mechanism of action is that MTX inhibits indirectly polyamine-producing enzymes. As a consequence, decreased polyamine production leads to increased

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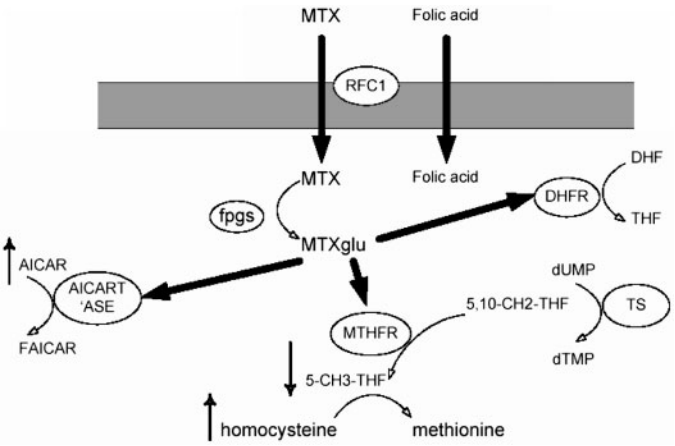


FIG. 1. Simplified representation of the folate pathway. Enzymes and metabolites involved in the folate pathway are shown. MTX inhibits cellular synthesis of purines, pyrimidines and methionine. MTX, methotrexate; MTXglu, methotrexate polyglutamate; RFC1, reduced folate carrier; FPGS, folylpolyglutamate synthetase; TS, thymidylate synthetase; DHFR, dihydrofolate reductase; DHF, dihydrofolate; THF, tetrahydrofolate; 5,10-CH2-THF, 5,10-methylene tetrahydrofolate; MTHFR, methylene tetrahydrofolate reductase; 5-CH3-THF, 5-methyl tetrahydrofolate; AICAR, 5-aminoimidazole-4-carboxamide-ribonucleotide; AICART'ASE, AICAR transformylase; FAICAR, formyl-AICAR. Reproduced, with permission from reference [6].

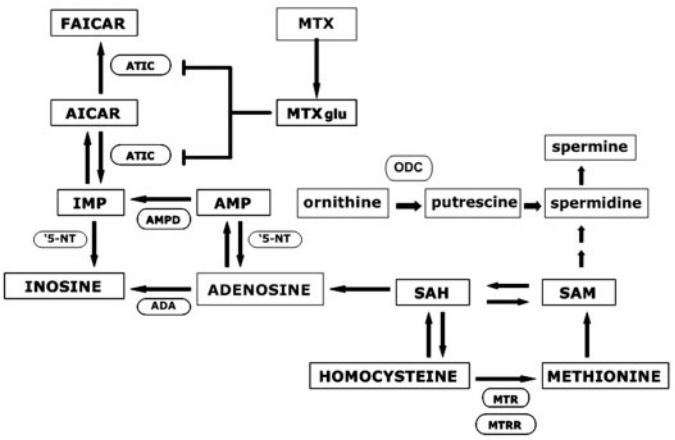


FIG. 2. Simplified representation of the adenosine metabolism and polyamine production pathway. Shown are enzymes and metabolites, involved in the step-wise release of adenosine and in the production of polyamines spermine and spermidine. ADA, adenosine deaminase; AICAR, 5-aminoimidazole-4 carboxamide ribonucleotide; AMP, adenosine monophosphate; AMPD, AMP-deaminase; FAICAR, formyl-AICAR; IMP, inosine monophosphate; MTR, methionine synthase; MTRR, methionine synthase reductase; MTX, methotrexate; MTXglu, methotrexate polyglutamate; SAH, s-adenosylhomocysteine; SAM, s-adenosylmethionine; ODC, ornithine decarboxylase; 5′-NT ecto-5′-nucleotidase.

intracellular ROS levels. In one study, it was shown that ODC overexpression reduces intracellular ROS and prevents loss of mitochondrial membrane potential. As a consequence, MTX-induced apoptosis was reduced [11]. In a second study, it was shown that MTX-induced apoptosis was prevented in a dose-dependent manner through prolactin, which increases ODC activity [12]. Conversely, in this study, it was shown that MTX alone induced apoptosis via ROS-dependent and mitochondria-mediated pathways, and led to clonal deletion of activated T cells.

In summary, MTX induces ROS in a time- and concentration-dependent manner. Higher ROS levels are found in active lymphocytes and may induce apoptotic response, whereas in monocytes, an adaptive response of cell proliferation inhibition is found.

Additional evidence for MTX-induced apoptosis was found through an increased activation-induced cell death and CD95 sensitivity, a member of the death receptor family [14]. After prolonged stimulation, activated T cells turned into a CD95-sensitive state with only CD45 + RO cells (activated T cells) exhibiting this increased sensitivity. MTX did not alter CD95 ligand expression. These data suggest that MTX may play a role in T-cell homeostasis through other mechanisms than ROS production.

Quemeneur *et al.* [13] studied the effects of micro-environmental elements on the anti-proliferative effects of MTX. These researchers compared purine and pyrimidine nucleotide depletion on primary T-cell proliferation and survival. It was shown that the inhibition of activated T-cell division occurred in a dose-dependent manner, mainly through blocking pyrimidine syntheses because only the addition of thymidine, not adenosine and guanosine, partially prevented the inhibitory effect of MTX on cellular divisions. In addition, the MTX-induced apoptosis upon T-cell activation and their data showed that apoptosis was increased with the number of T-cell divisions.

Surprisingly, a novel mechanism of the anti-proliferative effects of MTX was reported through the role of isoprenylcysteine carboxyl methyltransferase (ICMT) in human colon cancer DK0B8 cells [15]. It is known that MTX treatment increases homocysteine levels, which subsequently may increase s-adenosylhomocysteine (SAH) levels (Fig. 2). Consequently, SAH inhibits ICMT that is a methyltransferase. It was hypothesized that inhibition of ICMT via increased SAH levels may lead to reduced Ras protein methylation. The Ras protein is a central component in signal transduction pathways, regulating cell growth and differentiation. Winter-Vann *et al.* [15] showed a decrease of ~90% in Ras protein methylation with MTX treatment. Although the DK0B8 cell line expresses highly inducible K-Ras, these data suggested that ICMT inhibition is a critical component of the anti-proliferative effect of MTX through the reduction of Ras protein methylation. In addition, ICMT has more substrates than Ras protein, suggesting that other regulating genes or proteins may be (de)activated by methylation.

However, there is a clinical report suggesting that MTX is not a general anti-proliferative drug in RA. It was found that MTX induced specific clonal deletion of mononuclear cells of active RA patients and non-active RA patients [10]. Data showed that MTX reduced the predominant CD4+CD28+ subpopulation in active RA patients by 30% and the minor subpopulation of CD4+CD28− by 34% in active RA patients. The incidence of CD25 (IL-2 receptor) phenotype was downregulated by 15%. In contrast, in non-active RA patients, the CD4+CD28+ subpopulation appeared to be activated, whereas the CD4+CD28− was unaffected. These authors suggested that MTX might be more beneficial in active RA.

Even though several studies indicate that MTX induces apoptosis, there is only one report that relates the percentage of apoptotic peripheral blood mononuclear cells (PBMCs) after incubation with MTX to the American College of Rheumatology 20 response (ACR20) [18]. To obtain an ACR20 response, the number of swollen and tender joints has to reduce by 20% in combination with 20% improvement in three out of five other clinical endpoints. In this study, PBMCs of RA patients were activated with phytohaemagglutinin (PHA) and treated with MTX. No concentration-dependent increase of apoptotic cells and no relation of the percentage of apoptotic cells with clinical response defined as ACR20 response was seen. It was concluded that PBMC MTX-induced apoptosis was not a good predictor for optimizing MTX treatment.

In conclusion, inhibition of cell proliferation and apoptosis may be the result of multiple targets of MTX. The effects seem to be more profound in activated lymphocytes, with ROS production as the predominant underlying mechanism of action.

## Adenosine release

There are many reports showing that MTX directly or indirectly releases endogenous anti-inflammatory adenosine [6, 19–26]. Adenosine is a purine nucleoside that binds four specific adenosine receptors, A1, A2a, A2b and A3 [19]. These receptors differ in their affinity for adenosine and in their predominance on different cells and exert different effects on immunoregulation. For example, it is demonstrated that ligation of the A1-receptor leads to immunostimulation of neutrophils, whereas ligation of the A2a-receptor leads to immunosupression. However, it is hypothesized that the anti-inflammatory effects are predominantly due to A2a-receptor stimulation [19].

Recent data in adenosine A2a- and A3-receptor knockout mice provided further evidence that MTX acts through adenosine release [20]. The effects of MTX on acute inflammation were studied in an air-pouch model in A2a- and A3-receptor knockout and wild-type mice. It was shown that MTX reduced the leucocyte accumulation and TNF-$\alpha$ concentration in air-pouch exudates in the wild-type mice only, whereas the adenosine concentration increased 2- to 4-fold. Recently, these researchers provided additional support that MTX may act through adenosine release. Ecto-5′-nucleotidase (ecto-5′-NT) gene-deficient mice are unable to convert adenosine monophosphate (AMP) to adenosine extracellularly. In their animal arthritis model, ecto-5′-NT gene-deficient and wild-type mice were treated with injections of saline or MTX. As expected, MTX treatment reduced the number of leucocytes and TNF-$\alpha$ levels in the exudates and increased exudate adenosine concentrations in wild-type mice, whereas in ecto-5′-NT gene-deficient mice MTX did not cause any change [27].

Furthermore, adenosine receptor antagonism was shown to reduce MTX anti-inflammatory effects. Caffeine is a non-selective adenosine receptor antagonist, which has been proven to diminish MTX efficacy in inflammatory arthritis animal models [28]. Moreover, this study showed that selective adenosine receptor blockade was not sufficient to affect the capacity of MTX. However, the results of adenosine receptor blockade through caffeine on treatment outcome in clinical studies with patients treated with MTX are conflicting. Dietary caffeine has been associated with reduced efficacy of MTX [29], whereas others found no affect of MTX on efficacy [30]. Variability in caffeine consumption among patients and concomitant drug use to treat RA are probably reasons for these discrepancies in results. Therefore, it remains unclear whether adenosine antagonism via dietary caffeine is clinically relevant for MTX treatment outcome.

Although distinct adenosine receptor ligations may explain different effects, it is also demonstrated that genetic differences explain, at least partially, differences in resistance to the anti-inflammatory effects of MTX through adenosine [21]. One study compared four mouse models in their response upon MTX. In two mouse models, adenosine concentration was increased and leucocyte count was reduced in air-pouch exudates upon MTX treatment, whereas in two mouse models no effect of MTX was observed. These two mouse models probably failed to increase adenosine concentrations in response to MTX. Genetic mapping of the four mice identified loci containing candidate genes for which alleles that alter gene regulation or function could directly explain the response upon MTX.

In addition to genetic differences in adenosine release, metabolic enzyme activities involved in adenosine metabolism may account for reduced anti-inflammatory adenosine effects. The role of adenosine deaminase 1 (ADA1) and ADA2 isoenzymes was studied in RA and osteoarthritis patients [22].

ADA metabolizes adenosine into inosine, with ADA1 exerting a higher affinity for adenosine than ADA2 (Fig. 2). In this study, a higher ADA1 activity in RA synovial fluid than in osteoarthritis patients or RA patients' sera was observed. Thus, increased activity of ADA1 might reduce the anti-inflammatory effects of adenosine, subsequently MTX (Fig. 2). An *ex vivo* study already showed decreased enzyme activities of purine enzymes ADA, purine-nucleoside phosphorylase and hypoxanthine-guanine-phosphoribosyltransferase in mononuclear cells of RA patients after 48 weeks of MTX treatment [23]. These decreased enzyme activities were not influenced by folinic/folic acid use. No difference in activity of ecto-5′-NT, which converts AMP into adenosine was observed (Fig. 2). In conclusion, these clinical data suggest a favourable change in adenosine metabolism due to MTX treatment. However, in this cohort, no association between the enzyme activities and MTX efficacy and toxicity was found. Adenosine exhibits an extremely short half-life in serum; as a result, studies are hampered to monitor adenosine *in vivo* to resolve its association with treatment outcome. Previously, increased adenosine levels were successfully detected in patients treated with MTX and it was shown that MTX modulated the kinetics of adenosine in humans after 12 weeks of treatment [24]. It was shown that MTX inhibits ADA *in vivo* in RA patients. However, it was not clear whether this observed change was due to direct non-competitive enzyme inhibition, or decreased enzyme levels, or whether this reflected changes in lymphocyte subpopulations that could differ in their ADA activity. Moreover, it is not yet determined whether serum concentrations reflect synovial adenosine concentrations.

A remarkable effect of MTX-induced adenosine release was detected in a third study [25]. It was found that MTX significantly suppressed NURR1 expression via adenosine in patients with psoriatic arthritis. NURR1 is part of the NURR subfamily of orphan receptors within the steroid/thyroid receptor superfamily. Unlike most nuclear receptors, the NURR subfamily are products of immediate early genes, the expression of which can be induced in response to a variety of extracellular stimuli such as cytokines [25]. The NURR subfamily is known to regulate gene expression. In this study, a dose-dependent differential effect of MTX on steady-state and inducible NURR1 mRNA and protein levels was seen in primary synoviocytes and microvascular endothelial cells. Importantly, these authors showed that this effect of MTX is mediated through the adenosine receptor A2. It was also shown that adenosine alone mimicked the differential effects of MTX on NURR1 transcription. It was concluded that NURR1 is a molecular target of MTX action in inflammatory joint disease and demonstrated that the immunomodulatory actions of MTX on NURR1 were mediated through adenosine release.

However, the relation between adenosine and clinical efficacy of MTX needs to be explored. In one study, the association between adenosine concentration and MTX polyglutamation in erythrocytes in children with and without MTX treatment was assessed [26]. No significant correlations were found between adenosine concentration, MTX dose and MTX-polyglutamate concentration. The blood concentration of adenosine did not differ in patients in clinical responders when compared with non-responders, and the adenosine concentration did not differ between treated patients and controls. Yet, these findings may be the result of the technical difficulties in measuring adenosine concentrations *in vivo*.

In conclusion, the current results support the hypothesis that MTX modulates adenosine kinetics and dynamics, but the relationship between MTX dosage, MTX-polyglutamation and adenosine release and clinical effects are less well established.

## Cytokine levels and humoral responses

MTX was found to be an inhibitor of cytokine production induced by T-cell activation in whole-blood cultures of healthy donors and RA patients [31, 32]. MTX reduced the production of IL-4, IL-6, IL-13, TNF-$\alpha$, interferon gamma (IFN$\gamma$) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [31–33]. This inhibition of cytokines is suggested to be due to the *de novo* syntheses of purines and pyrimidines since the addition of folic acid, hypoxanthine and thymidine, guanosine or

adenosine reversed the inhibitory effects of MTX on cytokine production [31, 34]. It was shown that variable concentrations of MTX between donors were needed to inhibit cytokine production, whereas it was also shown that the inhibition depended on the activation stimulus [31]. In addition, cytokines produced by monocytes were hardly affected by MTX [31, 34].

Studying different cell lines, MTX did not affect lipopolysaccharide-induced cytokine IL-1$\beta$ or TNF-$\alpha$ release and production (mRNA) in monocytic cell line [35]. Interestingly, Cutolo *et al.* [36] suggested that the anti-inflammatory and anti-proliferative effects of MTX on differentiated monocytic myeloid cells are improved if the cells are pre-stimulated with testosterone but not with 17$\beta$-oestradiol. It could be the explanation for the supposed increased efficacy of MTX observed in males with RA.

However, *ex vivo* data from MTX-treated patients show that MTX treatment reduced TNF-$\alpha$ protein levels *in vivo* after 30 days, whereas *in vitro*, the difference in TNF-$\alpha$ levels after PHA-stimulation was no longer seen in comparison with controls after 180 days of therapy [37]. In addition, the TNF-$\alpha$ mRNA expression levels did not differ between patients and controls at any time point. It has to be noted that MTX in this study was used in combination with prednisolone.

In addition, the effect of MTX on *in vitro* spontaneous IL-6 and TNF-$\alpha$ production in whole-blood cultures of patients with juvenile RA, with and without treatment, and in healthy controls was studied [38]. There were no differences in the spontaneous production of cytokines between patients before and after 4 weeks of treatment with MTX, although after LPS stimulation, IL-6 levels were lower for patients treated with MTX when compared with patients receiving placebo. The authors concluded that MTX reduces IL-6 production in whole-blood cultures and therefore acts as an anti-inflammatory agent.

Further data on MTX action on cytokine production showed that MTX disrupted in a dose-dependent manner the interaction between synovial fibroblasts and T lymphocytes that favours synovial inflammation [39]. This decrease was due to inhibition of the upregulation of IL-15, IL-6, IL-8, CD69 (activation inducer molecule), CD25 (IL-2 receptor), IFN$\gamma$ and IL-17 in the co-culture of fibroblasts and T lymphocytes.

Others suggested that IL-1-driven disease is more responsive to MTX. In this study, cytokine levels in PBMCs from RA patients were related to MTX treatment and clinical outcome at 6 months [40]. Patients were categorized into four groups based on the response to MTX, as measured by the ACR criteria. IL1-receptor antagonist (IL1-Ra), IL1$\beta$, soluble TNF receptors p55 and p75, and TNF-$\alpha$ were measured. Good and excellent clinical responders (response >50% ACR improvement) associated with a significantly lower IL1-Ra/IL1$\beta$ ratio before treatment when compared according to their response status. The decreased ratios in most good responders were due to an enhanced constitutive IL1$\beta$ release. Much less marked, there was a slightly significant increase of soluble TNF receptors in the excellent responders (>70% ACR improvement). It was hypothesized that a highly inflammatory type of monocytes with a particular low IL1-Ra/IL1$\beta$ syntheses ratio is a prerequisite for MTX efficacy.

Humoral effects of MTX were studied on expression levels of activating receptors for IgG (Fc$\gamma$R) on monocytes of RA patients *ex vivo* and *in vitro* [41]. Triggering of Fc$\gamma$ receptors on monocytes initiates phagocytosis, antigen presentation, antibody-dependent cell-mediated cytotoxicity and release of pro-inflammatory and tissue-destructive cytokines such as IL1$\beta$, TNF-$\alpha$ and matrix metalloproteinases. Fc$\gamma$RI, Fc$\gamma$RIIA and Fc$\gamma$RIIIA are activating receptors, whereas Fc$\gamma$RIIB is an inhibitory receptor.

The study revealed an MTX-induced downregulation of Fc$\gamma$RI and Fc$\gamma$RIIA expression levels on monocytes in MTX-treated patients, which may thus prevent activation of monocytes/macrophages via immunocomplexes. The decrease in Fc$\gamma$RIIIA expression levels on monocytes was less marked. In addition, the percentage decrease in Fc$\gamma$RI expression correlated with the decrease in CRP and well-being.

The *in vitro* studies showed that MTX selectively decreased Fc$\gamma$RI and Fc$\gamma$RII, without decreasing the expression of CD40, CD80 and CD86 molecules. An effect of MTX on Fc$\gamma$RI (CD64) was also observed in a second study with RA patients [42]. It was found that MTX reduced Fc$\gamma$RI expression in leucocytes. However, no correlations with CRP and ESR were detected. These results show that MTX may reduce monocyte activity through decreasing the expression of activating receptors for IgG.

Although many *ex vivo* and *in vitro* data have been offered, the molecular basis for MTX-induced reduction of cytokine levels in most observations is unclear. An appealing pharmacological basis of MTX mechanism of action on cytokine production may be the suppression of activation of the NF-$\kappa$B signalling pathway.

TNF-$\alpha$ binding to TNFR1 activates the NF-$\kappa$B pathway. As a result, the transcription factor NF-$\kappa$B translocates to the cell nucleus and activates a wide range of genes. Previously, it was shown that MTX correlated with the inhibition of I$\kappa$B$\alpha$ degradation and the suppression of its phosphorylation [43]. As a result, NF-$\kappa$B stays in the cytoplasm and the NF-$\kappa$B signalling pathway is not activated. These molecular effects of MTX were at least partially attributed to the release of adenosine since ecto-5′-NT blocked and adenosine 2B receptor antagonism reversed the effect. Recently, NF-$\kappa$B-dependent gene expression of 34 genes in leucocytes of RA patients treated with anti-TNF-$\alpha$ agents and MTX showed a high correlation with disease activity before treatment, as measured by DAS [44]. Yet, there was no association between change in disease activity and RA treatment in this study.

In conclusion, MTX probably reduces the production of many cytokines including IL-4, IL-13, TNF-$\alpha$, IFN$\gamma$ and GM-CSF, IL-6 in activated cells, Fc$\gamma$RI and Fc$\gamma$RIIA expression levels. Furthermore, MTX inhibits the upregulation of IL-15, IL-8, CD69, CD25 and IL-17 and seems more beneficial for patients with a low IL1-Ra/IL1$\beta$ ratio before treatment. However, only a few studies have provided the molecular basis for their findings.

## Cellular adhesion molecules

The reduction of inflammation in RA may not only be due to apoptosis, reduction of cell proliferation and the inhibition of cytokine production but also through mechanisms affecting the expression of cellular adhesion molecules.

Cellular adhesion molecules (CAM) play an important role in the mediation of leucocyte–endothelial interactions, whereas leucocyte extravasations through the endothelial barrier are important in the pathogenesis of RA.

Generally, CAMs are classified in three supergene families known as integrin, immunoglobulin and selectin families. These supergene families play a distinct role in leucocyte emigration into the arthritic synovium [45].

Intracellular adhesion molecule-1 (ICAM-1) is a factor on endothelium that is involved in leucocyte adhesion but integrin factors such as ICAM-1 are also able to mediate cell–cell contacts [45]. Research showed that MTX suppressed ICAM-1 and lymphocyte-associated antigen (CLA) molecule expression in stimulated lymphocytes. This mechanism of action was found to be folate-and adenosine dependent for ICAM-1 and only folate dependent for CLA (Table 1) [46]. The factor CLA is a ligand for E-selectin on the endothelium, whereas E-selectin mediates the adhesion of cells to the endothelium.

Surprisingly, no effect of MTX was found on the expression of ICAM-1 and lymphocyte function-associated antigen (LFA, a receptor-counter part for ICAM-1) in a co-culture of fibroblasts and T lymphocytes of RA patients (Table 1) [39]. These researchers did observe a marked decrease in the number of lymphocytes adherent to fibroblasts. The effects of MTX on this cross-talk between T lymphocytes and fibroblasts were reversed by ADA, suggesting that adenosine release mediates the inhibition

TABLE 1. Leukocyte–endothelial adhesion molecules in relation to MTX treatment

| Factor | Effect of MTX on molecular adhesion molecule | Reference | Note |
|---|---|---|---|
| ICAM-1; CLA | Suppression of expression on T cells | [20] | Folate- and partially adenosine-dependent pathways |
| ICAM-1(CD54); LFA (CD11a) | No effect on up-regulated ICAM-1 expression in fibroblasts. No effect on LFA expression on T lymphocytes | [37] | Adenosine-dependent cross-talk in fibroblasts and T lymphocytes co-culture |
| CD3; CD4; CD8; CD68; E-selectin; ICAM-1; VCAM; MMP-3; TIMP-1 | Suppression of CD3, CD4, CD8, CD68, E-selectin, ICAM-1 expression, but not VCAM. MMP-3 mRNA expression in synovium is reduced, but not TIMP-1 mRNA | [42] | Psoriatic arthritis synovium biopsies |
| CLA; E-selectin; VLA-4; VCAM-1;CD25 | Decreases CLA expression on T cells, and down-regulation of E-selectin. No effect on VCAM-1, CD25 or VLA-4 expression | [43] | Psoriasis patients skin biopsies used |
| ICAM-1; E-selectin; VCAM-1 | Reduction of expression | [44] | Bullous pemphigoid patients skin biopsies used |
| ICAM-1; VCAM-1 | Suppression of TNF$\alpha$-induced expression | [45] | HUVEC cells used |
| PECAM-1; ICAM-3 | Suppression of expression | [46] | Psoriatic epidermis biopsies used |

ICAM-1/ICAM-3, intracellular adhesion molecule-1; CLA, cutaneous lymphocyte associated antigen; LFA, lymphocyte function-associated antigen; MMP-3, metalloproteinase-3; TIMP-1, metallopeptidase inhibitor 1; VLA-4, very late antigen-4; VCAM-1, vascular cell adhesion molecule-1; PECAM-1, platelet endothelial cell adhesion molecule.

of this synovial fibroblast–T lymphocyte cross-talk. Furthermore, the addition of an adenosine A2 receptor antagonist reversed the MTX effects.

Several studies involving inflammatory autoimmune diseases showed an inhibitory effect of MTX on several cellular adhesion molecules (Table 1). E-selectin and ICAM-1 expression seem to be consistently reduced by MTX, whereas MTX is suggested not to have an effect on vascular cell adhesion molecule (VCAM) expression in these studies [47–51]. For RA patients, it is suggested that suppressing IL-6 concentrations with DMARD treatment, including MTX, decreases endothelial activation, as determined by ICAM-1, endothelial leucocyte adhesion molecule (ELAM-1) and VCAM-1 expression [52].

In brief, MTX probably reduces CLA, E-selectin, ICAM-1 and -3, platelet endothelial cell adhesion molecule (PECAM-1) and VCAM-1 expressions in several inflammatory autoimmune diseases next to reducing cytokine levels. Current results in RA patients suggest that MTX may reduce cellular adhesion molecule expression; although it is not clear whether this is a direct effect or an indirect effect via the reduced expression of cytokines.

## Bone formation

MTX has been found to reduce progression of bone damage and therefore of functional decline [53]. To reveal this mechanism of MTX action, the interaction between PBMCs and RA fibroblasts (FLS) was studied in a co-culture. This culture is known to be capable of osteoclast formation in the presence of human macrophage colony-stimulating factor (M-CSF) and 1,25-dihydroxyvitamin $D_3$ [54]. It was found that MTX suppressed the expression of receptor activator of NF-$\kappa$B ligand (RANKL, an osteoclast differentiation factor) and RANKL mRNA, whereas MTX increased the secretion of osteoprotegerin (OPG, an osteoclastogenesis inhibitory factor). However, OPG mRNA levels and osteoclast differentiation factor receptor expression (RANK) in PBMCs were not altered. Therefore, these authors suggested that MTX inhibits osteoclast formation in a dose-dependent manner, probably due to the modulation of the RANKL:OPG ratio since no direct osteoclast cytotoxicty was detected.

A second study investigated the effect of MTX on the growth and differentiation of human cells of the osteoblast lineage [human bone-derived cells (HBDCs)] [55]. Alkaline phosphates and STRO-1, which is a trypsin-resistant cell surface antigen expressed by a subset of human marrow stromal cells, were chosen as cell development markers. MTX did not affect the expression of these markers. In addition, MTX did not alter the proliferation of HBDCs. Only in bone marrow stromal cells (BMSCs) was a decrease in the number of harvested cells found. It was concluded that MTX had no effect on the proliferation and maturation of osteoblast lineage, although MTX inhibited the proliferation of primitive bone marrow stromal cells, without affecting their osteogenetic differentiation. In addition, no effect of MTX on *in vivo* bone mineral density and bone turnover markers such as osteocalcin (bone formation marker), bone-specific alkaline phosphates and deoxypyridinoline (bone resorption markers) was observed [56].

Others showed that MTX suppressed IL-6 production in osteoblastic cell lines, after stimulation with several agonists [57]. No effect was shown without stimulation of osteoblastic cells. It was reported that IL-6 mRNA levels were not altered after incubation with MTX, suggesting that inhibition of IL-6 was due to inhibition at the protein level.

In conclusion, these results do not show that MTX has a direct cytotoxic effect on osteoclasts or osteoblasts, but MTX inhibited osteoclast formation indirectly, probably through modulation of the RANKL:OPG ratio or inhibition of IL-6 syntheses in osteoblasts, which attributes to the preventive effect of MTX on bone resorption by osteoclasts.

## Angiogenesis

Angiogenesis is a complex process through which new blood vessels grow from a pre-existing vasculature regulated by different soluble factors [58]. Vascular endothelial cell growth factor (VEGF), which is produced in higher amounts by local inflammatory effector cells in RA, and other factors such as basic fibroblast growth factor, cellular adhesion molecules are considered to contribute to angiogenesis in the rheumatic pannus.

The anti-angiogenesis effects of MTX were investigated in a placenta angiogenesis assay and in a collagen-induced arthritis (CIA) mice model [59]. Data showed that the spreading of microvessels from placental vessel fragments was not inhibited by MTX. Likewise, treatment with MTX of CIA in DBA/1 mice did not significantly reduce vessel growth. One group looked at the effect of MTX on gliostatin (GLS) and platelet-derived endothelial cell growth factor (PD-ECGF) expression in fibroblast synoviocytes obtained from RA patients. GLS is a protein factor that induces angiogenesis by a mechanism involving proliferation and chemotactic migration of endothelial cells. MTX did not have a significant influence on GLS/PD-ECGF mRNA expression or GLS protein levels [60]. However, others suggested, based on their findings in an *in vitro* model of pannus-like tissue, that MTX-induced pannus growth retardation could be contributed by the absence of angiogenesis, in addition to the inhibition of synoviocytes differentiation [61].

In conclusion, current data do not provide evidence that MTX affects angiogenesis. On the other hand, it may contribute to anti-angiogenesis effects through an indirect manner such as the

disruption of macrophage and fibroblast-like cell interaction or reduced cellular adhesion molecule expression.

## Discussion

MTX exerts a variety of pharmacological actions that are likely to account for its anti-proliferative and immunosuppressive effects in RA. Accordingly, clinical effects of MTX can be attributed to multiple targets. Recent studies indicate that MTX acts through direct promotion of cell apoptosis blocking proliferation of lymphocytes and monocytes, inhibiting cytokine production, influencing bone formation, probably reducing CAM expression and increasing extracellullar adenosine release.

It is likely that apoptosis is partly induced by MTX in highly activated T cells, whereas the other mechanisms mediate their effects by cell signalling pathways or inhibiting leucocyte migration to the synovium, leading to prolonged and sustained immunosuppression and anti-proliferation. The effects on monocytes seem to be less profound when compared with lymphocytes. Moreover, the fact that MTX modulated some animal models of RA, but not others, also indicates that MTX is not a general immunosuppressive agent. This may imply that MTX is probably effective in only specific molecular subgroups of RA [21, 62].

There is less evidence for a direct effect of MTX on angiogenesis and bone damage. On the other hand, MTX interrupts cell signalling, reduces cell proliferation and reduces CAMs, all of which contribute to pannus formation and osteoclast differentiation.

Obviously, extrapolation of the results from cell cultures is difficult because different cell lines, MTX concentrations, incubation times and activation stimuli are used. In addition, the heterogeneous effects of MTX are also due to genetic variation, interactions between cells and the differential effects of MTX and MTX-PG *in vitro* and *in vivo*. Moreover, outcomes of MTX may be mRNA expression or proteins levels, with or without polymorphisms influencing their functionality. Therefore, more molecular pharmacological studies involving MTX and MTX-PG in RA patients are needed to reveal the precise interactions between drug, drug target and disease.

Despite the discrepancies, there are many resemblances among the findings. Most reports find a dose-dependent and time-dependent effect of MTX. Many effects are reverted if folic/folinic acid or adenosine receptor blockers are used, indicating that MTX probably acts through the inhibition of pyrimidine and purine syntheses. Genetically based differences contribute to MTX efficacy, toxicity and resistance either in a direct manner, e.g. drug transporters or drug targets, or in an indirect manner, e.g. altered cytokine or natural folate levels.

Although RA has a broad spectrum of clinical manifestations, it might be that different molecular subtypes in RA share common pathways of response to the MTX [63]. Therefore, understanding the basic mechanism of MTX action may be useful in identifying those RA patients who are most likely to respond or most likely to experience a toxic response. However, to date, only a few reports relate the pharmacological actions of MTX to clinical parameters. Thus, the challenge for the future is the identification of markers that are relevant to the clinical response to MTX.

Our goal was to identify the various effects of MTX and to provide an update of the recent literature. This review may serve as a molecular basis in finding the most useful clinical markers for MTX efficacy in RA patients.

### Rheumatology key messages

- MTX exerts a variety of pharmacologic actions which account for its anti-proliferative and immunosuppressive effects
- The challenge is to link biological markers relevant to the response to MTX in RA

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