Low-dose Methotrexate Results in the Selective Accumulation of Aminoimidazole Carboxamide Ribotide in an Erythroblastoid Cell Line

Ryan S. Funk¹, Leon van Haandel¹, Mara L. Becker and J. Steven Leeder

Division of Clinical Pharmacology and Therapeutic Innovation, Children's Mercy Hospital, Kansas City, MO 64108

¹ Both authors contributed equally to this work.
Running Title:

Selective accumulation of ZMP with low-dose methotrexate

To whom correspondence may be addressed:

Ryan S. Funk, PharmD, PhD
Division of Clinical Pharmacology and Therapeutic Innovation
Children's Mercy Hospital
2401 Gillham Road
Kansas City, MO 64108
Tel: 816-234-9386
Fax: 816-855-1958
E-mail: rsfunk@cmh.edu

38 pages
3 tables
8 figures
53 references
242 words in the Abstract
729 words in the Introduction
1493 words in the Discussion
Nonstandard abbreviations:
AICART, aminoimidazole carboxamide ribonucleotide transformylase; ATase, amido phosphoribosyltransferase; BCA, bicinchoninic acid; CH₂-THF, methylene-tetrahydrofolate; D-PBS, Dulbecco’s phosphate buffered saline; DHF, dihydrofolate; DHF-PGₙ, n-glutamate form of dihydrofolate; DHFR, dihydrofolate reductase; dUMP, deoxyuridine monophosphate; EC₅₀, concentration resulting in 50% of maximal response; f-THF, formyl-tetrahydrofolate; FA, folic acid; FGAR, formyl-glycinamide ribonucleotide; GAR, glycinamide ribonucleotide; GART, phosphoribosylglycinamide formyltransferase; Kᵢ, enzyme inhibition constant; Kᵣ, half-maximal saturation constant; MTX, methotrexate; MTX-PGₙ, n-glutamate form of methotrexate; MTX-PGₜₒₜᵃˡ, sum total of methotrexate and its polyglutamates; PA, peak area; PRPP, phosphoribosyl pyrophosphate; RFC, reduced folate carrier; SRM, Selected Reaction Monitoring; THF, tetrahydrofolate; TS, thymidylate synthase; ZMP, aminoimidazole carboxamide ribotide.

Section:
Inflammation, Immunopharmacology and Asthma
Abstract

Therapeutic and toxic response to low-dose methotrexate (MTX) in the treatment of autoimmune disease continues to be highly variable resulting in a critical need to identify predictive biomarkers of response. Biomarker development has been hampered by an incomplete understanding of the molecular pharmacology of low-dose MTX. To address this issue, the accumulation of the substrates for aminoimidazole carboxamide ribonucleotide transformylase (AICART) and thymidylate synthase (TS) were measured as markers of pharmacological activity of MTX in an erythroblastoid cell line. A 115-fold increase in the AICART substrate and anti-inflammatory mediator, aminoimidazole carboxamide ribotide (ZMP), was observed following exposure to 10 nM MTX, but subsequently decreased with increasing MTX concentrations, declining to baseline levels with 1000 nM MTX. In contrast, the TS substrate, deoxyuridine monophosphate (dUMP), displayed concentration-dependent accumulation, increasing 29-, 342- and 471-fold over baseline with 10, 100 and 1000 nM MTX, respectively. Cellular levels of dUMP correlated with levels of parent drug (MTX-PG1) (r=0.66, p<0.001) and its polyglutamates (MTX-PG2-6) (r=0.81, p<0.001), whereas, cellular levels of ZMP were only moderately correlated with MTX-PG1 (r=0.34, p<0.01). In contrast, accumulation of ZMP at 10 nM MTX was associated with a 2.9-fold increase in of the AICART inhibitor dihydrofolate (DHF), represented primarily by long-chain DHF polyglutamates. Selectivity, defined as the ratio of ZMP to dUMP, was maximal following exposure to 6 nM MTX. Characterizing the range of MTX concentrations that selectively promote ZMP accumulation while preserving pyrimidine biosynthesis may lead to optimization of low-dose MTX therapy.
Introduction

Low-dose methotrexate (MTX) has become a commonly used therapeutic option as an anti-inflammatory in the treatment of various autoimmune diseases, including rheumatoid arthritis, lupus, psoriasis and juvenile idiopathic arthritis (Weinblatt et al., 1985; Giannini et al., 1992; Weinblatt et al., 1994; Fortin et al., 2008; Kalb et al., 2009). Despite its widespread use, response to low dose MTX therapy is highly variable and currently unpredictable with failure to achieve disease remission occurring in 40% or more of adult and pediatric arthritis patients (Lambert et al., 2004; Ruperto et al., 2004). Hepatic, gastrointestinal and hematological toxicities are commonly reported and often therapy limiting (Weinblatt, 1985; Becker et al., 2010). In addition, several months of therapy are often required before response can be accurately judged. For patients that fail to respond to MTX therapy, this delay in disease control results in potential disease progression and irreversible joint damage. Thus, early identification of clinical biomarkers that predict optimal response or risk of toxicity will have important clinical implications.

Recent investigations have included genetic, pharmacokinetic and pharmacodynamic markers of response (Halilova et al., 2012). Notably, the use of erythrocyte measurements of MTX and its polyglutamates has been proposed to reflect both a pharmacokinetic and a pharmacodynamic metric for clinical use (Dervieux et al., 2004; Dervieux et al., 2005). The importance of the erythrocyte as a pharmacokinetic component of this measurement is reflected in the short plasma half-life of MTX that makes plasma measurements impractical (Kremer et al., 1986). The pharmacodynamic hypothesis is based on the notion that the polyglutamates of MTX are the biologically
active form of the drug and may, therefore, be predictive of pharmacologic activity. Metabolism of MTX to its polyglutamated forms has been found to result in increasingly potent inhibition of enzymes of nucleotide biosynthesis, including: thymidylate synthase (TS), phosphoribosylglycinamidine formyltransferase (GART), aminoimidazole carboxamide ribonucleotide transformylase (AICART), and amido phosphoribosyltransferase (ATase) (Allegra et al., 1985a; Allegra et al., 1985b; Baram et al., 1988; Sant et al., 1992). However, such a correlation between MTX polyglutamation and inhibition of these enzymes in a cellular system has not been experimentally evaluated.

To date, biomarker development has been hampered by an incomplete understanding of the mechanism of action of low-dose MTX (Chan and Cronstein, 2010). The anti-proliferative and anti-inflammatory effects of MTX are believed to be distinct, occurring through the inhibition of different biochemical pathways. Specifically, the anti-proliferative activity of MTX is primarily attributed to inhibition of purine and pyrimidine nucleotide biosynthesis through depletion of intracellular reduced folate cofactors, via inhibition of dihydrofolate reductase (DHFR), and direct inhibition of TS and enzymes of de novo purine biosynthesis, including: ATase, GART and AICART (Borsa and Whitmore, 1969b; Pinedo et al., 1976). In addition, inhibition of DHFR by MTX results in the cellular accumulation of dihydrofolate (DHF), which also has varying potency in inhibition of these enzymes (Allegra et al., 1985b; Allegra et al., 1987; Baram et al., 1988; Chu et al., 1990; Sant et al., 1992). The anti-inflammatory mechanism of MTX activity remains controversial, but current evidence supports the inhibition of the purine synthesis pathway through direct inhibition of AICART (Figure 1) (Baggott et al.,
1998; Baggott et al., 1999; Morgan et al., 2004; Baggott and Morgan, 2007). Inhibition of AICART has been found to result in the accumulation of aminomimidazole carboxamide ribotide (ZMP) and its metabolites, which inhibit adenosine deaminase and AMP deaminase resulting in an increase in adenosine and adenine nucleotide levels (Cronstein et al., 1991; Baggott et al., 1993; Morabito et al., 1998). Elevated levels of extracellular adenosine, through adenosine receptor activation, results in a reduction in inflammation and may depend on MTX polyglutamate formation (Baggott et al., 1986; Cronstein et al., 1993; Urakawa et al., 2000; Dolezalova et al., 2005; You et al., 2013). Selective toxicity towards T lymphocytes, and activation of AMP-activated protein kinase (AMPK) by ZMP have also been proposed as mechanisms of activity of MTX in autoimmune disorders (Taisun and Baggott, 1994; Genestier et al., 1998; Fairbanks et al., 1999; Johnston et al., 2005; Beckers et al., 2006; Katerelos et al., 2010).

Despite experimental evidence that MTX is a potent inhibitor of purified AICART, no study has thoroughly evaluated the exposure-dependent accumulation of ZMP in a cellular system following exposure to MTX. Therefore, a major focus of this study was to understand the relationship between low-dose MTX and the accumulation of this potentially important biochemical in mammalian cells.

**Materials and Methods**

**Cell Culture.** K562 cells (erythroblastoid, human, GM05372) were obtained from Coriell Cell Respository (Camden, NJ) and grown in RPMI-1640 medium (Life Sciences, 61870-127) supplemented with 10% fetal bovine serum (Atlanta Biologicals, S11150). Unless otherwise stated cells were maintained under normal growth conditions in a 37
°C and 5% CO2 controlled incubator at a density between 2 x 10⁵ and 1 x 10⁶ cells/mL to maintain cells in a logarithmic growth phase. All experiments were conducted within 6 passages following removal from cryopreservation.

Cell Growth/Cytotoxicity Studies. K562 cells under normal growth conditions were exposed to 1000 nM MTX and monitored over a 96 hour period for changes in cell growth and viability as determined by the trypan blue exclusion assay and routine cell counting on a hemocytometer. In addition, cell growth and cytotoxicity was measured using reagents provided in the Live/Dead® Viability/Cytotoxicity kit (Invitrogen, L-3324). Briefly, K562 cells seeded into a 96-well plate were treated with MTX concentrations between 1 nM and 30 mM in triplicate for 24 hours under normal growth conditions. Live cell controls consisted of untreated cells and dead cell controls consisted of cells pre-treated with 70% ethanol. Following MTX treatment, calcein-AM and ethidium homodimer-1 were added to each well at a final concentration of 4 μM and incubated at room temperature for 45 minutes. Fluorescence signal was determined for each sample using a Biotek Synergy HT fluorescence plate reader equipped with excitation/emission filters set at 485 nm/528 nm and 530 nm/645 nm. Percent viability and cytotoxicity was determined as the fluorescence signal relative to the untreated control.

MTX Treatment. K562 cells under normal culture conditions were counted using a hemocytometer and for each experiment a sample of approximately 2.5 x 10⁶ cells was obtained for baseline measurements and denoted the zero hour sample. Following resuspension at a density of 2.5 x 10⁵ cells/mL in fresh growth media, a volume of 5 mL/well was added to 6-well cell culture grade polystyrene plates. MTX (Schircks
Laboratories, #16.411) in D-PBS, or D-PBS alone (untreated control), was added to each well from sterile-filtered 100x stock solutions. Each sample was mixed gently and maintained under normal growth conditions for up to 72 hours. Cell samples containing approximately 1 x 10^5 to 5 x 10^6 cells were obtained through routine sampling at specified time points and washed in duplicate with 1 mL of 4°C D-PBS. A 25 μL aliquot was removed preceding the second wash step and retained for analysis of cellular protein content using the micro BCA method (Thermo Scientific, 23235). The remaining cellular pellet was stored at -80°C prior to analysis.

**Sample preparation.** Cell samples were removed from -80 °C storage and maintained on ice during processing. Frozen cell pellets were re-suspended in 50 μL extraction buffer consisting of 40% acetonitrile, 40% methanol and 20% 0.1M phosphate buffered ultra pure water at pH 7.4 containing 0.1% 2-mercaptoethanol and 1% sodium ascorbate. Samples were vortexed for 30 seconds and centrifuged at 16,100 x g for 3 minutes, to remove proteins. The resulting supernatant was subsequently analyzed by UPLC/MS/MS.

**Analytical Methodology.** MTX and its polyglutamated metabolites were measured according to an earlier published procedure involving ion-pair UPLC/MS/MS (van Haandel et al., 2009; van Haandel et al., 2011). DHF polyglutamates were measured by UPLC/MS/MS using an appended version of a previously described method (van Haandel et al., 2012). ZMP, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5’-monophosphate (Toronto Research Chemicals, A611705), and dUMP, 2’-deoxyuridine 5’-monophosphate disodium salt (Sigma, D3876), were measured with an additional UPLC/MS/MS assay. Separation was conducted on a Waters BEH HILIC
chromatography column (100 x 2.1 mm packed with 1.7 μM particles). A linear gradient with an isocratic hold for 0.2 minutes at 2.5% mobile phase A, 70% A at 2.2 minutes, and 2.5% A at 2.3 minutes with a re-equilibration time of 2 minutes was utilized to elute the analytes. Mobile phase A consisted of 20 mM ammonium acetate (pH 10.0) and mobile phase B consisted of acetonitrile with a flow rate of 0.3 mL/minute. The mass spectrometer was equipped with an electrospray ionization source operating in negative ion mode. Selected Reaction Monitoring (SRM) was utilized to detect ZMP (337 → 79) and dUMP (307 → 195), and collision energies were 15 and 20 V respectively. Quantitation occurred by interpolation of the sample signal on a 5 point calibration curve ranging from 0.1 to 100 μM constructed for each analyte. Measured ZMP and dUMP concentrations were normalized for protein content and expressed in peak area (PA) or fmol per microgram of cellular protein.

**Results**

**Cellular uptake of MTX and its polyglutamate metabolites.** The choice of the K562 cell line in these studies was based on two criteria, 1) the erythroblastoid properties of the cells (Andersson et al., 1979) with respect to the current use of erythrocyte MTX content as a biomarker of MTX toxicity and response, and 2) the documented capacity of K562 cells to actively transport (Matherly et al., 1991) and polyglutamate (Koizumi, 1988) MTX. Preliminary studies with qualitative analysis were conducted to determine the optimum range of MTX concentrations and exposure times for subsequent quantitative analysis.
Uptake of MTX in the K562 cell line has been reported to be primarily mediated through the reduced folate carrier (RFC) with a half-maximal saturation constant ($K_t$) of 4.64 $\mu$M (Matherly et al., 1991), which is well above the maximum concentration of 1000 nM used in this study. Under these conditions, cellular uptake of MTX was linear and concentration dependent over the 24 hour period with mean ($\pm$ SD) uptake velocities of 0.76 ($\pm$0.13), 1.62 ($\pm$0.49) and 7.78 ($\pm$2.75) PA/$\mu$g protein/hr following exposure to 10, 100 and 1000 nM MTX, respectively (Figure 2). Total MTX uptake at the end of the 24 hour exposure was found to be 18 ($\pm$ 3), 39 ($\pm$ 11) and 189 ($\pm$ 62) PA/$\mu$g protein for the 10, 100 and 1000 nM MTX treated cells, respectively. Significant cellular toxicity was not observed with MTX concentrations of up to 1000 nM for 24 hr as determined by trypan blue exclusion and analysis with the fluorescent probes calcein-AM and ethidium homodimer-1 fluorescence (Supplemental Figure 1).

As the pharmacological activity of MTX is also dependent on the formation of polyglutamate metabolites because of their increased cellular retention and inhibitory activity against several target enzymes (Jolivet et al., 1982), the cellular accumulation of the parent compound, MTX (i.e. MTX-PG$_1$), and its polyglutamated metabolites, containing between two (i.e. MTX-PG$_2$) and six (i.e. MTX-PG$_6$) glutamate residues was also determined over the 24 hour drug exposure (Figure 3). The initial rate of MTX-PG$_1$ uptake was rapid with higher extracellular MTX concentrations (100 and 1000nM) with near steady state levels achieved within one hour. In contrast, when exposed to an extracellular concentration of 10nM MTX, uptake of MTX-PG$_1$ continued to increase throughout the treatment period. At the end of the treatment period, MTX-PG$_1$ levels
varied by at most 3-fold and were found to be 14.2 (±2.2), 7.7 (±1.4) and 24.5 (±7.9) PA/µg protein for the 10, 100 and 1000 nM MTX treated cells, respectively.

In contrast to the parent compound, the accumulation of MTX polyglutamates was found to display a higher degree of dependence on MTX concentration and exposure time. MTX-PG₂ and MTX-PG₃ were detected with all three MTX treatment concentrations and varied by as much as 9- and 200-fold, respectively. MTX-PG₄ and MTX-PG₅ were only detected at the higher two concentrations of MTX, with an 8-fold difference observed between the two concentrations for both species. MTX-PG₆ was only detected following the 24 hour exposure to the highest concentration of MTX (Figure 3). At the end of the treatment period, mean (±SD) concentrations of the polyglutamated species (i.e. MTX-PG₂-₆) were found to be 3.4 (± 0.9), 31.6 (± 10.1) and 164.7 (±54.6) PA/µg protein for the 10, 100 and 1000 nM MTX treated cells, respectively. Further analysis of the MTX polyglutamate distribution was conducted by determining the percentage of total MTX that was accounted for by each polyglutamate species (Figure 4). Of note, MTX-PG₁ accounted for 81.0 (± 1.9), 20.1 (± 3.5) and 13.1 (±1.5) % of total cellular MTX in the 10, 100 and 1000 nM MTX treated cells, respectively. In contrast, higher order MTX polyglutamates (MTX-PG₂-₆) accounted for 19.0 (± 1.9), 79.9 (± 3.6) and 86.9 (± 1.5) % of total cellular MTX in the 10, 100 and 1000 nM MTX treated cells, respectively.

Accumulation of substrates for TS and AICART. The concentration- and time-dependent inhibition of TS and AICART was monitored by measuring intracellular levels of their respective substrates, ZMP and dUMP (Figure 5). Levels of dUMP increased with increasing concentrations of MTX in a time-dependent manner.
Following a 24 hour exposure to 10, 100 and 1000 nM MTX, dUMP reached levels of 1327 (±83), 15511 (±3952) and 21404 (±2213) PA/μg protein, respectively, representing a 29-, 342- and 471-fold increase over untreated control levels of 45 (±9) PA/μg protein, reflecting concentration-dependent inhibition of TS. In contrast, intracellular ZMP levels did not display the same exposure-dependent response. In the initial 8 hours of drug exposure, ZMP levels appeared mostly unchanged under all treatment conditions. However, after 24 hours of exposure ZMP levels in the 10, 100 and 1000 nM MTX samples measured 2253 (±199), 103 (±34) and 19 (±8) PA/μg protein, respectively. As compared to untreated control ZMP measurements of 19 (±11) PA/μg protein, these measurements represent a 115- and 5-fold increase in ZMP levels following the 10 and 100 nM MTX treatments, respectively, and no change in levels following the 1000 nM MTX.

Pearson pair-wise comparisons between the cellular accumulation of dUMP or ZMP and the accumulation of MTX and its polyglutamates were conducted (Table 1). Intracellular levels of dUMP, significantly correlated with total uptake of MTX (MTX-PG<sub>total</sub>), however this was not observed for ZMP. In addition, further analysis showed that intracellular levels of dUMP significantly correlated with each of the measured polyglutamate species, most strongly observed with MTX-PG<sub>3</sub> (r=0.81, p<0.001). In contrast, ZMP levels were only moderately correlated with levels of the parent drug (MTX-PG<sub>1</sub>) (r=0.34, p<0.01) and not to any of the measured polyglutamate species.

**Time- and concentration-dependent accumulation of ZMP and dUMP.** Given the discordance between MTX effects on ZMP and dUMP accumulation, subsequent studies were designed to elucidate the response over a narrower concentration range
with longer exposure times. K562 cells were exposed to MTX concentrations ranging from 1 to 100 nM and untreated control (0 nM MTX) for up to 72 hours. Intracellular levels of ZMP, dUMP and the ratio of the analytes were determined both as a function of concentration and time (Figure 6). Analysis was conducted on a calibrated analytical system allowing for absolute quantification of ZMP and dUMP.

In agreement with the previous set of experiments, ZMP levels were maximal following exposure to 10 nM MTX, and were sustained throughout the 72 hour exposure, reaching levels 170-fold greater than the untreated control (4421 vs. 26 fmole/μg protein). At 5 nM MTX, ZMP levels progressively increased with time reaching a level of 523 fmole/μg protein that was 20-fold greater than control values. In contrast, MTX concentrations greater than 10 nM resulted in an initial increase in ZMP concentrations that subsequently returned to control levels over the 72 hour period of exposure.

Intracellular dUMP levels were also highly responsive to MTX concentration and exposure time. A slight increase in dUMP was observed at the 10 nM MTX concentration reaching levels up to 40-fold greater than the untreated control (1128 vs. 52 fmole/μg protein). The maximum response was observed at 25 nM MTX (15633 fmole/μg protein) and represented a 620-fold increase over control. Interestingly, at concentrations greater than 10 nM MTX, dUMP concentrations continued to increase, reaching a maximum at 48 hr and declining thereafter.

To assess the relative effect of low concentrations of MTX on ZMP and dUMP accumulation, the ratio of intracellular ZMP to dUMP was also determined. Although the maximum ZMP accumulation was observed at the 10 nM MTX concentration,
maximum selectivity for inhibition of AICART relative to TS occurred at 5 nM MTX with a
ZMP:dUMP ratio of 14.3, representing a 48-fold increase in selectivity over the
untreated baseline value of 0.3. At 10 nM MTX a favorable ratio of up to 4.4 was
observed, representing a 15-fold increase in selectivity. However, selectivity is
continued to decrease at higher MTX concentrations due to both a decrease in ZMP
accumulation and a pronounced increase in dUMP levels with selectivity ratios as low
as 0.004, 75-fold lower than the untreated control, and 3575-fold lower than the
maximum observed selectivity at 5 nM MTX.

**Fine-scale optimization of ZMP accumulation.** To determine the
concentration of MTX that provides the maximum selectivity for accumulation of ZMP
without significant inhibition of TS in K562 cells, cells were exposed to 12 different MTX
concentrations between 0.5 and 20 nM and untreated control (0 nM) for 24 hr. Replicate
experiments were conducted on three separate days (Figure 7).

In support of the previous findings, ZMP levels increased in a concentration-
dependent fashion reaching levels of 4032 (±326) fmole/μg protein in the 10 nM MTX
treated cells, representing a 194-fold increase over the untreated control levels of 21 (±
6) fmole/μg protein. ZMP levels remained significantly elevated after exposure to the 15
and 20 nM MTX concentrations; however, a trend towards declining ZMP levels was
apparent at these higher MTX concentrations. The data were fit to a 3-parameter Hill
equation and the effective concentration resulting in 50% of the maximal response
(EC50) was determined to be 6.1 (±1.2) nM. Maximal increase in dUMP was observed
with the highest tested concentration (i.e., 20 nM MTX) reaching 2878 (±1196) fmole/μg
protein and represents a 129-fold increase over control levels of 22 (± 14) fmole/μg
protein. Selectivity, expressed as the ratio of ZMP to dUMP concentrations was maximal following exposure to 6 nM MTX, with a 36-fold increase in the ZMP to dUMP ratio.

**DHF polyglutamate accumulation at low MTX concentrations.** To determine the effect of low concentrations of MTX on cellular levels of DHF, K562 cells were exposed to 10 nM MTX for 24 hours and the changes in cellular DHF were determined. MTX caused a significant increase in DHF to levels 2.9-fold greater than the untreated controls (Figure 8A). Furthermore, MTX exposure resulted in a shift in DHF polyglutamation to long-chain polyglutamates, with the weighted average glutamate distribution shifting from a mean (±SD) of 5.52 (±0.05) to 5.95 (±0.08) glutamate residues per molecule of DHF (p < 0.01) (Figure 8B). This effect was most pronounced for DHF-PG₆, which was increased 4.3-fold over control.

**Discussion**

In this study, exposure of erythroblastoid cells to low concentrations of MTX resulted in the accumulation of the AICART substrate and anti-inflammatory mediator, ZMP. Accumulation of ZMP did not correlate with the formation of MTX polyglutamates and was observed at concentrations below those necessary for inhibition of TS. Together, these findings substantiate the selective accumulation of ZMP with low-dose MTX and do not support a relationship between levels of MTX polyglutamates and ZMP. However, the finding that DHF accumulates at low MTX concentrations may be indicative of its role in the selective accumulation of ZMP. These results will be discussed in regard to the biochemical pharmacology of MTX and pharmacodynamic
markers of MTX activity, with therapeutic implications in the context of the hypothesized role of ZMP as the mediator of the anti-inflammatory effects of low-dose MTX therapy.

Intact cellular transport and metabolism of MTX is evidenced by the kinetics of MTX accumulation (Figure 2) and polyglutamte formation (Figure 3). MTX accumulation was time and concentration dependent with no evidence of saturation. The initial accumulation of MTX-PG₁ appears to reach a rapid equilibrium consistent with high-affinity binding of MTX-PG₁ (Goldman, 1974), with subsequent accumulation occurring primarily through the formation of higher order glutamates. This is perhaps best illustrated by the formation of MTX-PG₂ in the 10 nM MTX treated cells only once MTX-PG₁ approached saturating levels after 8 hours. Further formation of the higher-order polyglutamates (MTX-PG₃-₆) didn’t appear to display the same saturation limited formation, but were highly dependent on MTX exposure. The MTX polyglutamate distribution at the end of the 24 hour treatment differed with MTX concentration, MTX-PG₁ being the predominate species following the 10 nM MTX treatment, compared to the predominately polyglutamated species (MTX-PG₂-₆) following the 100 and 1000 nM MTX treatments (Figure 4).

Drug activity was measured through accumulation of substrates for the MTX-targeted enzymes, AICART and TS. Despite the ability of MTX to inhibit various enzymes, we focused on AICART and TS to explore the balance of the hypothesized anti-inflammatory and anti-proliferative effects of MTX. ZMP is the proposed mediator of the anti-inflammatory effects of MTX, whereas, the TS substrate, dUMP, is a byproduct of inhibition of pyrimidine synthesis, which is important for the anti-proliferative activity of MTX (Borsa and Whitmore, 1969a). The elevated ZMP
concentrations observed only after exposure to low concentrations of MTX (Figures 5-7)
may be explained by a selective inhibition of AICART relative to other upstream
enzymes in the purine biosynthesis pathway (Figure 1), including ATase and GART
(Lyons and Christopherson, 1991). These findings suggest that ZMP measurements
may not provide a useful marker for inhibition of purine biosynthesis, but may provide an
important biochemical marker for the anti-inflammatory effects of MTX. In vivo, levels of
ZMP and its metabolites have been observed to increase intracellularly in splenocytes
of MTX treated mice, as well as in animal and human biological fluids with MTX therapy,
but a relationship with therapeutic response has not been established (Cronstein et al.,
1993; Baggott et al., 1999; Smolenska et al., 1999; Baggott and Morgan, 2007). Further
in vivo clinical studies are needed to define the relationship between ZMP and the anti-
inflammatory activity of MTX before its value as a biomarker can be determined.

Although MTX polyglutamates are potent inhibitors of AICART in purified enzyme
preparations (Allegra et al., 1985b), cellular levels of MTX polyglutamates were not
significantly associated with ZMP levels in the cellular system used in this current study
(Table 1). In an intact cellular system, MTX and its polyglutamates as well as
endogenous folate inhibitors, such as DHF, would be expected to not only inhibit
AICART, but also upstream enzymes in the purine biosynthesis pathway as illustrated in
Figure 1, providing an explanation for the poor correlation between cellular MTX
polyglutamate and ZMP concentrations.

Although modest, the correlation between the accumulation of MTX-PG1 and
ZMP suggests that the parent compound may be active in the inhibition of AICART
(Table 1). However, it can be seen that low levels of MTX-PG2, MTX-PG3 and
saturating levels of MTX-PG₁ (Figure 3) correspond to increased levels of ZMP at the 10 nM MTX concentration (Figure 5A). This observation suggests that short-chain polyglutamates or saturation of high-affinity binding sites (i.e. DHFR) by MTX-PG₁ contributes to the accumulation of ZMP. The former would be in accordance with recent findings that clinical response in rheumatoid arthritis correlates with erythrocyte levels of MTX-PG₂ (Hobl et al., 2012). Although the relationship between ZMP accumulation and therapeutic response remains to be established, the finding that ZMP accumulates without significant MTX polyglutamate formation suggests that efforts to measure cellular MTX polyglutamates as a predictor of therapeutic response may not be predictive of ZMP accumulation.

Selective inhibition of AICART by MTX or its short-chain polyglutamates is not supported by the reported inhibition constants (Kᵢ) for these compounds (Table 2). Specifically, MTX-PG₁, MTX-PG₂ and MTX-PG₃ are 11-, 32 and 4-fold more potent inhibitors of TS than AICART, and therefore, low concentrations of MTX would be expected to have an observable effect on dUMP accumulation prior to any detectable effect on ZMP. In contrast, DHF-PG₅ is reported to be 12-fold more potent as an inhibitor of AICART compared to TS, supporting DHF as being responsible for the observed concentration-dependent selectivity of ZMP accumulation at low MTX concentrations (Table 3). The observed accumulation of DHF, predominately in the form of DHF-PG₆, following exposure to low MTX concentrations (Figure 8) would support the hypothesized role of DHF-polyglutamates in the selective inhibition of AICART (Figure 7).
In contrast to ZMP, the cellular accumulation of dUMP correlates with the cellular accumulation of MTX and its polyglutamated metabolites (Table 1). In agreement with the reported $K_i$ values (Table 2), dUMP accumulation correlates more strongly with MTX polyglutamates than with cellular levels of the parent drug. Given the complexity of a cellular system, it may be possible that TS is directly or indirectly inhibited by MTX and its polyglutamates. However, the strong relationship between polyglutamate formation and dUMP accumulation does provide evidence to support the role of MTX polyglutamates in the direct inhibition of TS. Therefore, in contrast to AICART, clinical measurements of intracellular MTX polyglutamates would be expected to correspond with inhibition of TS and may represent a potential biomarker for inhibition of the pyrimidine synthesis pathway and its downstream effects.

The pronounced accumulation of ZMP despite a minimal increase in dUMP after prolonged exposure to low concentrations of MTX suggests that low drug doses can be used to selectively target the accumulation of the anti-inflammatory mediator without significantly inhibiting TS activity (Figure 7). Hence, sustained exposure to low levels of MTX may result in improved anti-inflammatory activity without the effects associated with inhibition of pyrimidine biosynthesis. Clinically, these findings would suggest that therapeutic response may also be paradoxically related to dose with prolonged exposure to lower doses resulting in more favorable response due to the selective accumulation of ZMP in conditions where this result is aimed for, such as inflammatory arthritis. Supportive of this concept, stepwise increases in MTX dose in JIA patients who were non-responders to standard low dose MTX did not result in improved clinical outcomes (Ruperto et al., 2004).
In conclusion, the data presented in this study indicates that increases in external MTX concentration is associated with cellular accumulation of MTX over time, and is accompanied by a shift toward higher order MTX polyglutamation. As the external MTX concentration increases to approximately 10 nM, ZMP accumulates due to selective inhibition of AICART. As the external MTX concentration increases beyond 20 nM, ZMP accumulation declines, consistent with inhibitory effects of MTX on other cellular targets, including enzymes upstream of AICART in the purine biosynthesis pathway that would restrict ZMP formation as well as inhibition of TS characterized by increased accumulation of its substrate, dUMP, and decreased production of the product of the methylation reaction, DHF. In K562 cells, selective formation of ZMP was optimal at 6 nM, reflecting preferential inhibition of AICART at lower MTX concentrations while preserving TS activity. Increasing MTX beyond this point results in decreased formation of anti-inflammatory species and increased risk of toxicity due to inhibition of other cellular folate-dependent pathways. These data have clinical implications for the use of low-dose MTX as an anti-inflammatory agent. First, the data imply that the relative abundance of ZMP and dUMP may support their use as biomarkers for characterizing the potential for efficacy and toxicity of low-dose MTX. Second, the dose of MTX required to optimize the ZMP/dUMP ratio in individual patients may vary according to their unique genetic constitution and interactions with environmental factors, such as dietary folate and changing biological demands for folate. Additional work is required to replicate our findings in additional cell lines, and to determine the functional consequence of allelic variation in key folate pathway genes to provide insight into the role of genetic variation on optimization of MTX treatment in patients with inflammatory
conditions. An improved understanding of the complex biochemical pharmacology of MTX will prove paramount in the identification and evaluation of therapeutic biomarkers in the future, and may pave the way for individualization of therapy.
Authorship Contributions

Participated in research design: Funk, van Haandel, Becker and Leeder.

Conducted experiments: Funk and van Haandel.

Performed data analysis: Funk and van Haandel.

Wrote or contributed to the writing of the manuscript: Funk, van Haandel, Becker and Leeder.
References


Baggott JE, Vaughn WH and Hudson BB (1986) Inhibition of 5-aminoimidazole-4-carboxamide ribotide transformylase, adenosine deaminase and 5'-adenylate deaminase by polyglutamates of methotrexate and oxidized folates and by 5-aminoimidazole-4-carboxamide riboside and ribotide. *The Biochemical journal* **236:**193-200.


Immunosuppressive properties of methotrexate: apoptosis and clonal deletion of

Giannini EH, Brewer EJ, Kuzmina N, Shaikov A, Maximov A, Vorontsov I, Fink CW,
rheumatoid arthritis. Results of the U.S.A.-U.S.S.R. double-blind, placebo-
controlled trial. The Pediatric Rheumatology Collaborative Study Group and The
Cooperative Children's Study Group. The New England journal of medicine
326:1043-1049.

Goldman ID (1974) The mechanism of action of methotrexate. I. Interaction with a low-
affinity intracellular site required for maximum inhibition of deoxyribonucleic acid
synthesis in L-cell mouse fibroblasts. Molecular pharmacology 10:257-274.

Halilova KI, Brown EE, Morgan SL, Bridges SL, Jr., Hwang MH, Arnett DK and Danila
MI (2012) Markers of treatment response to methotrexate in rheumatoid arthritis:

Hobl EL, Jilma B, Erlacher L, Duhm B, Mustak M, Broll H, Hogger P, Rizovski B and
Mader RM (2012) A short-chain methotrexate polyglutamate as outcome
parameter in rheumatoid arthritis patients receiving methotrexate. Clinical and
experimental rheumatology 30:156-163.

Johnston A, Gudjonsson JE, Sigmundsdottir H, Ludviksson BR and Valdimarsson H
(2005) The anti-inflammatory action of methotrexate is not mediated by
lymphocyte apoptosis, but by the suppression of activation and adhesion


juvenile idiopathic arthritis who failed to respond to standard doses of methotrexate. *Arthritis and rheumatism* **50**:2191-2201.


van Haandel L, Becker ML, Williams TD, Stobaugh JF and Leeder JS (2012)


Footnotes

This work was supported in part by the Children’s Mercy Hospital and Clinics; the American College of Rheumatology, Research and Education Foundation Rheumatology Investigator Award; and the National Institutes of Health Eunice Kennedy Shriver National Institute of Child Health and Human Development [Grant T32-HD069038].
Figure Legends

**Figure 1.** A diagram of the hypothesized biochemical basis for the anti-proliferative and anti-inflammatory activities of MTX. Inside the cell MTX and its polyglutamate metabolites bind with high affinity to DHFR inhibiting the reduction of folic acid (FA) and DHF to tetrahydrofolate (THF) resulting in depletion of the reduced folate pool, including: THF, methylene-tetrahydrofolate (CH₂-THF) and formyl-tetrahydrofolate (f-THF). Inhibition of enzymes of nucleotide biosynthesis, including: ATase, GART, AICART and TS, are inhibited through, 1) depletion of their respective reduced folate cofactors, 2) direct inhibition by MTX and its polyglutamate metabolites and 3) direct inhibition by DHF. The anti-proliferative activity of MTX is attributed to inhibition of purine and pyrimidine biosynthesis, whereas the anti-inflammatory activity is hypothesized to occur through inhibition of AICART resulting in the accumulation of its substrate and anti-inflammatory mediator, ZMP.

**Figure 2.** Kinetic analysis of MTX accumulation in an erythroblastoid cell line. K562 cells maintained under normal culture conditions were treated with MTX at extracellular concentrations of 10, 100 and 1000 nM for 0, 1, 2, 4, 8, and 24 hrs. Cellular levels of MTX and its polyglutamate metabolites (MTX-PG_{total}) were determined by liquid chromatography-tandem mass spectrometry and normalized to cellular protein content. MTX-PG_{total}, expressed in PA/μg protein, is represented as mean ± SD from three independent experimental evaluations. Data were fit by linear regression and the resulting slopes were expressed as the calculated rate of MTX-PG_{total} accumulation under each treatment condition.
Figure 3. Kinetic analysis of cellular accumulation of MTX and its polyglutamate metabolites. K562 cells were exposed to MTX at extracellular concentrations of 10, 100 or 1000 nM for 0, 1, 2, 4, 8, and 24 hrs under normal culture conditions. Cellular levels of parent drug (i.e. MTX-PG₁) and each polyglutamate metabolite (i.e., MTX-PG₂ through MTX-PG₆) were determined by liquid chromatography-tandem mass spectrometry and expressed in PA/μg protein. Each sample is represented as the mean ± SD from three independent experimental evaluations.

Figure 4. Effect of MTX concentration on cellular MTX polyglutamate distribution. Following a 24 hr exposure to MTX at concentrations of 10, 100 and 1000 nM, the percent of total cellular MTX represented by parent drug (i.e. MTX-PG₁), each of its polyglutamate metabolites (i.e. MTX-PG₂ through MTX-PG₆) and the sum total of the polyglutamate metabolites (MTX-PG₂-6) was determined. Each sample is represented as the mean ± SD from three independent experimental evaluations.

Figure 5. Kinetic analysis of accumulation of substrates for AICART and TS following exposure to MTX. K562 cells were exposed to MTX at extracellular concentrations of 0, 10, 100 or 1000 nM for 0, 1, 2, 4, 8, and 24 hrs under normal culture conditions. Cellular levels of the AICART substrate, (A) ZMP, and the TS substrate, (B) dUMP, were determined by liquid chromatography-tandem mass spectrometry. Analyte levels, expressed in PA/μg protein, are represented as the mean ± SD from three independent experimental evaluations.

Figure 6. Surface plots of ZMP and dUMP accumulation following exposure to MTX. K562 cells under normal culture conditions were exposed to extracellular MTX concentrations of 0, 10, 25, 50, 75 and 100 nM for 0, 24, 48 and 72 hrs. Cellular levels...
of (A) ZMP and (B) dUMP were determined by liquid chromatography-tandem mass spectrometry and expressed in fmole/μg protein. (C) The ratio of cellular ZMP to dUMP, termed selectivity, was determined for each sample.

**Figure 7.** Concentration-dependent selectivity for ZMP and dUMP accumulation with low concentrations of MTX. K562 cells under normal culture conditions were exposed to extracellular MTX concentrations of 0, 0.5, 1, 2, 4, 5, 6, 7, 8, 10, 15 and 20 nM for 24 hrs and cellular levels of (A) ZMP and (B) dUMP were determined by liquid chromatography-tandem mass spectrometry and expressed in fmole/μg protein. (C) Selectivity, defined as the ratio of cellular ZMP to dUMP was determined for each sample. Data are represented as the mean ± SD from three independent experimental evaluations.

**Figure 8.** DHF accumulation and polyglutamate distribution with low concentrations of MTX. K562 cells maintained under normal culture conditions were treated with or without 10 nM MTX for 24 hrs. Cellular levels of (A) DHF (DHF_{total}) and (B) each of its polyglutamated forms (DHF-PGn) were determined by liquid chromatography-tandem mass spectrometry, normalized to cellular protein content and expressed in PA/μg protein. Data are represented as mean ± SD from three independent experimental evaluations (*, p < 0.05; **, p < 0.01 by Student’s t test).
Table 1. Pearson’s pair-wise correlations (r-values) for the relationship between intracellular MTX polyglutamates and levels of ZMP and dUMP in MTX treated K562 cells.

<table>
<thead>
<tr>
<th>$K_i$ ($\mu$M)</th>
<th>MTX-PG&lt;sub&gt;total&lt;/sub&gt;</th>
<th>MTX-PG&lt;sub&gt;1&lt;/sub&gt;</th>
<th>MTX-PG&lt;sub&gt;2&lt;/sub&gt;</th>
<th>MTX-PG&lt;sub&gt;3&lt;/sub&gt;</th>
<th>MTX-PG&lt;sub&gt;4&lt;/sub&gt;</th>
<th>MTX-PG&lt;sub&gt;5&lt;/sub&gt;</th>
<th>MTX-PG&lt;sub&gt;6&lt;/sub&gt;</th>
<th>MTX-PG&lt;sub&gt;2-6&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZMP</td>
<td>-0.01</td>
<td>0.34**</td>
<td>-0.02</td>
<td>-0.08</td>
<td>-0.07</td>
<td>-0.07</td>
<td>-0.14</td>
<td>-0.07</td>
</tr>
<tr>
<td>dUMP</td>
<td>0.81***</td>
<td>0.66***</td>
<td>0.75***</td>
<td>0.81***</td>
<td>0.79***</td>
<td>0.78***</td>
<td>0.69***</td>
<td>0.81***</td>
</tr>
</tbody>
</table>

**, p < 0.01; ***, p < 0.001
Table 2. Inhibition constants ($K_i$) of enzymes involved in nucleotide biosynthesis for MTX and its polyglutamates (Allegra et al., 1985a; Allegra et al., 1985b; Drake et al., 1987; Baram et al., 1988; Sant et al., 1992).

<table>
<thead>
<tr>
<th>$K_i$ (µM)</th>
<th>MTX-PG₁</th>
<th>MTX-PG₂</th>
<th>MTX-PG₃</th>
<th>MTX-PG₄</th>
<th>MTX-PG₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHFR</td>
<td>$11.0 \times 10^{-5}$</td>
<td>$17.0 \times 10^{-5}$</td>
<td>$8.0 \times 10^{-5}$</td>
<td>−</td>
<td>$8.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>TS</td>
<td>13.0</td>
<td>0.17</td>
<td>0.14</td>
<td>0.13</td>
<td>0.047</td>
</tr>
<tr>
<td>AICART</td>
<td>143.9</td>
<td>5.47</td>
<td>0.56</td>
<td>0.056</td>
<td>0.057</td>
</tr>
<tr>
<td>GART</td>
<td>80</td>
<td>57</td>
<td>7.1</td>
<td>5.1</td>
<td>2.5</td>
</tr>
<tr>
<td>ATase</td>
<td>ND*</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>550</td>
</tr>
</tbody>
</table>

*, No inhibition was detected (ND).
Table 3. Inhibition constants (K_i) of enzymes involved in nucleotide biosynthesis for DHF and DHF-PG_5 (Allegra et al., 1985b; Baram et al., 1988; Sant et al., 1992).

<table>
<thead>
<tr>
<th></th>
<th>DHF-PG_1</th>
<th>DHF-PG_5</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS</td>
<td>77.7</td>
<td>0.5</td>
</tr>
<tr>
<td>AICART</td>
<td>63.3</td>
<td>0.043</td>
</tr>
<tr>
<td>GART</td>
<td>25.3</td>
<td>21.9</td>
</tr>
<tr>
<td>ATase</td>
<td>312</td>
<td>3.41</td>
</tr>
</tbody>
</table>
Figure 1

Pyrimidine Nucleotides

MTX DHF

dUMP

CH₂-THF

TS

PRPP

MTX DHF

ATase

GAR

MTX DHF

GART

FGAR

ZMP

AICART

Anti-inflammatory Response

Purine Nucleotides

MTX DHF

MTX DHF

DHFR

THF

f-THF

MTX

FA₁ DHFR
Figure 2

MTX-PG<sub>total</sub> (PA/μg protein)

- 10 nM MTX
- 100 nM MTX
- 1000 nM MTX

Time (hr)

0 5 10 15 20 25
Figure 3

MTX-PG1

MTX-PG2

MTX-PG3

MTX-PG4

MTX-PG5

MTX-PG6

10 nM MTX
100 nM MTX
1000 nM MTX

MTX-PGn (PA/µg protein)

Time (hr)

0 5 10 15 20 25

0.1 1 10 100

MTX-PGn (PA/µg protein)

Time (hr)

0 5 10 15 20 25

0.1 1 10 100
Figure 4

![Graph showing the percentage of MTX total for different concentrations of MTX and n (# glutamate residues).]
Figure 5

A

0 nM MTX
10 nM MTX
100 nM MTX
1000 nM MTX

ZMP
(PA/µg protein)

Time (hr)

B

0 nM MTX
10 nM MTX
100 nM MTX
1000 nM MTX

dUMP
(PA/µg protein)

Time (hr)
Figure 7

A

B

[Graph showing the relationship between MTX (nM) and Analyte (fmole/μg protein) for ZMP and dUMP, with error bars.]

[Graph showing the relationship between MTX (nM) and Selectivity (ZMP:dUMP) with error bars.]
Figure 8

A

\[
\text{DHF}_{\text{total}} \quad (\text{PA/\mu g protein})
\]

\begin{align*}
0 \text{ nM} & \quad 10 \text{ nM} \\
\end{align*}

B

\[
\text{DHF-PGn} \quad (\text{PA/\mu g protein})
\]

\begin{align*}
0 \text{ nM MTX} & \quad 10 \text{ nM MTX} \\
1 & \quad 2 & \quad 3 & \quad 4 & \quad 5 & \quad 6 & \quad 7 & \quad 8
\end{align*}