Published in final edited form as:

Arthritis Rheum. 2009 August ; 60(8): 2257–2261. doi:10.1002/art.24685.

Methotrexate catabolism to 7-hydroxy methotrexate in rheumatoid arthritis alters drug efficacy and retention and is reduced by folic acid supplements

Joseph E. Baggott, Ph.D.¹ and Sarah L. Morgan, M.D., R.D, C.C.D.^{1,2}

¹Department of Nutrition Sciences, The University of Alabama at Birmingham

²Department of Medicine, The University of Alabama at Birmingham

Abstract

Objective—Urinary excretion of methotrexate (MTX) and its catabolite 7-hydroxy-MTX (7-OH-MTX) were measured in two 24-hour collections after MTX therapy in patients with rheumatoid arthritis. The effect of folic and folinic acid supplements on this catabolism was determined in patients and *in vitro*. The effect of this catabolism on MTX efficacy and *in vivo* retention was determined.

Methods—Urine samples were collected after 6 and 7 weeks of therapy. MTX and 7-OH-MTX concentrations were determined by a high performance liquid chromatography mass spectroscopy. Swelling (SW) and pain/tenderness (P/T) indices were measured before and at 6 and 7 weeks of therapy. Patients received either folic or folinic acid supplements (1mg/day) from week 6 to 7.

Results—Folic acid not folinic acid inhibited aldehyde oxidase (AO), the 7-OH-MTX producing enzyme. Excretion of 7-OH-MTX as % of dose or mg 7-OH-MTX / g creatinine was not normally distributed (n = 39). Patients with marked improvement in SW and P/T indices had lower mean 7-OH-MTX excretion (p < 0.05). Folic acid supplementation lowered 7-OH-MTX excretion (p = 0.03). Relatively high 7-OH-MTX excretion was correlated (p < 0.05) with relatively high MTX excretion and with relatively low *in vivo* MTX retention (n = 35).

Conclusion—Non-normal distribution of 7-OH-MTX excretion suggests at least two phenotypes for this catabolism. Lower 7-OH-MTX formation suggests folic acid inhibition of AO and a better clinical response. Higher 7-OH-MTX formation may interfere with MTX polyglutamylation and binding to enzymes and therefore increase MTX excretion, decrease *in vivo* MTX retention and efficacy.

The methotrexate (MTX) catabolite, 7-hydroxymethotrexate (7-OH-MTX), is less efficacious in rat adjuvant arthritis than MTX (1) and this is possibly true in patients with RA. We reported that the percentage of an oral MTX dose excreted in 72-hour urine as 7-OH-MTX varied by a factor of 14 in patients with RA and that the distribution was bimodal (2). This suggested that patients with a relatively high capacity to catabolize MTX to 7-OH-MTX would have a less efficacious response. This catabolism is extensive; since the concentration of marrow 7-OH-MTX is 3 to 10 times that of MTX 24-hours after low-dose oral methotrexate (3). Thus, urinary excretion of 7-OH-MTX may be a surrogate for proportionately higher tissue levels.

We show here that folic acid (pteroylglutamic acid) competitively inhibits rabbit liver aldehyde oxidase (AO) (EC 1.2.3.1) and that folinic acid (5-formyl-tetrahydrofolic acid) doesn't strongly inhibit this enzyme. AO catalyzes the catabolism of MTX to 7-OH-MTX (4). These findings suggest that patients treated with MTX plus folic acid would have less catabolism of MTX to 7-OH-MTX compared to patients treated with MTX plus folinic acid.

Since 7-OH-MTX (like MTX) is polyglutamylated, high tissue levels of 7-OH-MTX could displace MTX from the active site of the polyglutamyl synthetase (EC 6.3.2.27) and increase urinary MTX excretion and reduce the *in vivo* retention MTX (5).

We tested the following questions:

- 1) Is the distribution of 7-OH-MTX excreted in the urine of patients with RA non normal?
- 2) Is MTX less efficacious in patients with a high capacity to catabolize it to 7-OH-MTX? 3) Does folic acid treatment (but not folinic acid treatment) reduce MTX to 7-OH-MTX catabolism? 4) Does the extent of this catabolism vary and is the amount of the MTX dose retained *in vivo* also altered by this catabolism?

Materials and Methods

Folic acid, folinic acid and MTX were purchased from Sigma. Rabbit liver AO was purified from cytosol as the 30-50% ammonium sulfate saturation fraction (4). The hydroxylation of MTX to 7-OH-MTX was assayed spectrophotometrically (4). Enzyme kinetic data was analyzed using the EZ-fit program (E.I. DuPont de Nemours, Wilmington, DE).

This protocol received Institutional Review Board approval. The demographic and clinical characteristics of the patients have been previously described (6). A 24-hour urine was collected immediately after the 6th weekly dose of MTX (visit 2) and the 7th weekly dose of MTX (visit 3). Patients received either daily folic acid doses or folinic acid doses (1mg/day, respectively) during week 7. Urine volumes were measured and aliquots were adjusted to pH=3 and stored at -70° C. Urine samples were located for 39 patients completing visit 2 and for 35 patients completing both visit 2 and visit 3 (17 receiving folic and 18 receiving folinic acid supplements). PPD Industries (Richmond, VA) measured MTX and 7-OH-MTX concentrations using a FDA-validated HPLC MS/MS assay. It is unlikely that MTX or 7-OH-MTX concentrations would change during the 5-6 years of storage due to the low storage temperature and acidification. Total MTX and 7-OH MTX excreted could be calculated from urine volumes, as could the mg or % of the dose excreted in urine or the average amount of the MTX dose retained in 24 hours, which assumes that the only loss is through urinary excretion. Creatinine concentrations were also determined (6).

The patients were evaluated for joint swelling (SW), pain and tenderness (P/T) as previously described (6). The disease activity score (DAS-28) was not calculated because all components were not available.

The Kolmogorov-Smirnov (KS) test was used to test for a normal distribution. Chi Square, linear regression (r), t tests and ANOVA followed by Scheffe's test were used where appropriate. The average percent dose (average of visit 2 and 3) excreted as 7-OH-MTX was log transformed before ANOVA to normalize the data.

Results

The K_m for MTX was 115 (± 18) μM and the K_i for folic acid was 72 (± 15) μM . Folinic acid in concentrations of 10 fold greater than MTX concentrations of 10 to 50 μM failed to inhibit the enzyme by more than 10%. Folic acid was a competitive inhibitor.

The mean (median) excretion of 7-OH-MTX as mg 7-OH-MTX /g of creatinine and as percent of MTX dose excreted as 7-OH-MTX were 0.291 mg/g (0.226 mg/g) and 2.85% (2.32%) respectively at visit 2. As shown in figures 1A and 1B the data distribution was not normal ($p = 0.01$, KS test) and appeared bimodal or multimodal. The mean ($\pm$ S.D.) MTX dose was 9.2 (± 2.2) mg ($n=10$) and 10.0 (± 2.0) mg ($n=9$), ($p>0.05$, t test) in patients

excreting less than 0.15 and greater than 0.45 mg of 7-OH-MTX / g creatinine, respectively. The mean ($\pm$ S.D.) MTX dose was 8.8 ($\pm$ 2.2) mg (n=12) and 9.3 ($\pm$ 2.2) mg (n=11), ($p>0.05$, t test) in patients excreting less than 1.8 % and greater than 3.6 % of the dose as 7-OH-MTX respectively. Therefore, the amount of 7-OH-MTX excreted was not correlated with the MTX dose.

The percentage of the MTX dose excreted as 7-OH-MTX was averaged for visit 2 and 3 for 35 patients and is presented in Table 1. The lowest amount of 7-OH MTX formed was in the group with marked improvement of joint indices. The mean MTX dose was 8.3 ($\pm$ 1.8) mg and 9.1 ($\pm$ 1.3) mg ($p>0.05$, t test) in patients with marked improvement and no change, respectively. Therefore MTX response was not related to the dose.

A comparison of paired urine samples before (visit 2) and after (visit 3) either folic acid or folinic acid supplementation was made with respect to mg 7-OH-MTX excreted / g creatinine. The number of patients in the folic acid treatment group in the increased, decreased or unchanged categories was 3, 7 and 7, respectively, and in the folinic acid treatment group it was 11, 3 and 4, respectively ($p = 0.03$, chi square). More patients treated with folic acid supplements had decreased or unchanged urinary 7-OH-MTX excretion than those treated with folinic acid supplements.

Data from visits 2 and 3 revealed that when there was an increase in mg 7-OH-MTX / g creatinine excreted at visit 3 there was also an increase in mg MTX / g creatinine excreted at visit 3 and visa versa. The change in these parameters (visit 3 minus visit 2) for both MTX and 7-OH-MTX in the same patient were plotted (Figure 2A). These changes were positively correlated ($r = 0.71$, $p<0.01$). This suggested that the more MTX catabolized to 7-OH-MTX the more MTX was excreted and therefore the less MTX retained *in vivo*. This change in average MTX retained *in vivo* was verified when the change in the % of the dose excreted as 7-OH-MTX (visit 3 minus visit 2) was plotted versus the change in average μ g of the MTX dose retained after 24 hours /kg body weight (visit 3 minus visit 2) (Figure 2B). The r value was -0.66 ($p<0.01$) indicating that the more 7-OH-MTX excreted the less of the MTX dose was retained in the 24 hours post dose.

Discussion

Folic acid, but not folinic acid, inhibited rabbit liver AO. Di and trinuclear heterocyclic aromatic compounds and folic acid, celecoxib, and prednisone have been reported to inhibit human liver AO *in vitro* (7). Our data suggests that folic acid binds to rabbit liver AO tighter than MTX (i.e. lower K_i for folic acid than K_m for MTX).

The capacity to catabolize MTX to 7-OH-MTX varied considerably between patients. We had reported that the percent of dose excreted as 7-OH-MTX varied from 0.94 to 13.2% in patients with RA in a 72-hour urine (2). The catabolic capacity was not a function of body/organ mass since the urinary excretion of 7-OH-MTX corrected for creatinine varied by a factor of 10 and was bimodal (Figure 1B). Others have reported variable capacity to catabolize MTX to 7-OH-MTX in patients with RA (references in 2) including a report that 2 of 16 patients had levels of 7-OH-MTX below detection limits (i.e. 1×10^{-9} M) in serum following oral MTX dosing (8). Variability in the *in vitro* activity of human liver AO has been reported (references in 2) including a 48-fold range of activities in human liver cytosols (9). Inhibitors of AO and xanthine oxidase (i.e. allopurinol) both reduce the formation of 7-OH-MTX in human liver cytosol (10). It is possible that both enzymes catalyze the formation of 7-OH-MTX.

Patients with a lower capacity to catabolize MTX to 7-OH-MTX also respond better to MTX. Our clinical data was collected after 6 and 7 weeks of MTX therapy and response

may have improved with therapy duration. We could not calculate the DAS-28 score which is a better measure of efficacy, which limits the interpretation of these results.

Folic acid supplements lower the *in vivo* formation of 7-OH-MTX when compared to folinic acid supplements. This may be due to inhibition of AO, since with dihydrofolate reductase inhibited by MTX; folic acid should be relatively trapped as such *in vivo*. On the other hand, folinic acid can be metabolized to other folate coenzymes independent of the reductase and folinic acid and its tetrahydrofolate metabolites have little effect on AO. Polyglutamylation of folic acid may lower its inhibition of AO (4), but daily dosing may maintain a pool of folic acid as the monoglutamate.

The more 7-OH-MTX excreted, the more MTX is excreted in the same individual (Figure 2A). We previously observed that when MTX catabolism to 7-OH-MTX is reduced by cyclosporine, the amount of MTX excreted was also reduced, although it was not statistically significant (11). This suggests that 7-OH-MTX competes with MTX for protein binding sites. One critical site would be the polyglutamyl synthetase. If relatively large amounts of 7-OH-MTX are formed, they may be polyglutamylated and sequestered inside cells at the expense of MTX which is excreted in greater quantities. The data in Figure 2B confirms that the more 7-OH-MTX excreted in 24-hours, the less of the MTX dose is retained during the 24 hours after the dose.

We hypothesize that there is a phenotype which produces relatively large amounts of 7-OH-MTX which excludes MTX from the polyglutamyl synthetase, forms more 7-OH-MTX polyglutamates (7-OH-MTX Glu_n), and excretes relatively more MTX and 7-OH-MTX in urine. A second phenotype produces relatively little 7-OH-MTX and 7-OH-MTX Glu_n and relatively little MTX and 7-OH-MTX is excreted in urine and retains more MTX Glu_n.

This, the consequences of excessive 7-OH-MTX formation would be not only to reduce the *in vivo* MTX pool but also to promote MTX excretion, lower *in vivo* MTX retention and possibly its efficacy. Others have reported that concentrations of MTX polyglutamates in red blood cells of patients after 6 months of therapy was variable and that lower concentrations were found in patients with a poorer clinical response (12). It has been reported in ten patients that one had no detectable pentaglutamates of MTX in red blood cells after 40 weeks of therapy and in nine others the time to detect this polyglutamate ranged from 1 to 28 weeks of therapy (13). The variability of red blood cell MTX polyglutamate concentrations could be a function of MTX catabolism to 7-OH-MTX. 7-OH-MTX could also displace MTX from the active site of other folate metabolizing enzymes (14), reducing the amount of MTX retained. The changes plotted in Figures 2A and 2B use the patients as their own control, thus, 7-OH-MTX formation and *in vivo* MTX retention can change in the same patient in one week. With stable MTX doses, it is likely that the patients' *in vivo* AO activity could be altered by changes in normobiotic activators or inhibitors, nutrients, amounts of other medications and changes in the amount of AO. .

In conclusion, the urinary excretion of 7-OH-MTX and the extent of catabolism of MTX to 7-OH-MTX were not normally distributed in patients and this catabolism is likely to alter MTX efficacy and the amount of the MTX dose retained *in vivo*. Folic acid supplements may inhibit *in vivo* AO and therefore the catabolism of MTX to 7-OH-MTX and potentiate the efficacy of MTX. There may be three reasons to use folic acid supplements in MTX treated patients with RA: 1) lower MTX toxicity, 2) lower blood homocysteine, and 3) reduce 7-OH-MTX formation.

Acknowledgments

We thank Dr. Kerry Lok for preparing the figures, Dr. Rui Feng for assistance with statistical analysis and Ms. Connie Bonds for preparing the manuscript.

Grant Support: Supported in part by GCRC Grant M01 RR-00032 from the National Center for Research Resources and the National Institute of Arthritis and Musculoskeletal and Skin Diseases and the Office of Dietary Supplements (1R29-AR-42673)

References

1. Baggott J, Morgan S, Koopman W. The effect of methotrexate and 7-OH-methotrexate on rat adjuvant arthritis and on urinary aminoimidazole carboxamide excretion. *Arthritis Rheum.* 1998; 41:1407–10. [PubMed: 9704638]
2. Baggott JE, Bridges SL, Morgan SL. Evidence for two phenotypes in the metabolism of methotrexate to 7-hydroxymethotrexate in patients with rheumatoid arthritis. *Arthritis Rheum.* 2005; 52:356–8. [PubMed: 15641087]
3. Sonneveld P, Schultz FW, Nooter K, Hählen K. Pharmacokinetics of methotrexate and 7-hydroxymethotrexate in plasma and bone marrow of children receiving *low*-dose oral methotrexate. *Cancer Chemother Pharmacol.* 1986; 18:111–116. [PubMed: 3791556]
4. Fabre GR, Seither R, Goldman ID. Hydroxylation of 4-amino-antifolates by partially purified aldehyde oxidase from rabbit liver. *Biochem Pharmacol.* 1986; 35:1325–30. [PubMed: 2421732]
5. Fabre G, Goldman ID. Formation of 7-hydroxymethotrexate polyglutamyl derivatives and their cytotoxicity in human chronic myelogenous leukemia cells, *in vitro*. *Cancer Res.* 1985; 45:80–5. [PubMed: 2578101]
6. Morgan SL, Oster RA, Lee JY, Alarcón GS, Baggott JE. The effect of folic acid and folinic acid supplements on purine metabolism in methotrexate-treated rheumatoid arthritis. *Arthritis Rheum.* 2004; 50:3104–11. [PubMed: 15476202]
7. Obach RS, Huynh P, Allen MC, Beeham C. Human liver aldehyde oxidase: *Clin Pharmacol.* 1993; 35:409–12.
8. Shiozawa K, Tanada Y, Yoshihara R, Imura S, Murata M, Yamane T, et al. Serum levels and pharmacodynamics of methotrexate and its metabolite 7-hydroxy methotrexate in Japanese patients with rheumatoid arthritis treated with 2-mg capsule of methotrexate three times per week. *Mod Rheumatol.* 2005; 15:405–9. [PubMed: 17029103]
9. Kitamura S, Sugihara K, Nakatani K, Ohta S, Ohhara T, Ninomiya S, Green CE, Tyson CA. Variation of hepatic methotrexate 7-hydroxylase activity in animals and humans. *IUBMB Life.* 1999; 48:607–11. [PubMed: 10683765]
10. Chládek J, Martínková J, Sispera L. An *in vitro* study on methotrexate hydroxylation in rat and human liver. *Physiol Res.* 1997; 46:371–9. [PubMed: 9728483]
11. Fox RI, Morgan SL, Smith HT, Robbins BA, Choc MG, Baggott JE. Combined oral cyclosporine and methotrexate therapy in patients with rheumatoid arthritis elevates methotrexate levels and reduces 7-hydroxymethotrexate levels when compared with methotrexate alone. *Rheumatology.* 2003; 42:989–94. [PubMed: 12730515]
12. Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. *Arthritis Rheum.* 2006; 54:3095–3103. [PubMed: 17009228]
13. Dalrymple JM, Stamp LK, O'Donnell JL, Chapman PT, Zhang M, Barclay ML. Pharmacokinetics of oral methotrexate in patients with rheumatoid arthritis. *Arthritis Rheum.* 2008; 58:3299–3308. [PubMed: 18975321]
14. Baggott JE, Morgan SL, Vaughn WH. Differences in methotrexate and 7 hydroxymethotrexate inhibition of folate-dependent enzymes of purine nucleotide biosynthesis. *Biochem J.* 1994; 300:627–9. [PubMed: 8010943]

Figure 1. Distribution of (A) the percent of the MTX dose excreted as 7-OH-MTX and (B) the mg 7-OH-MTX / g creatinine both in 24 hour urines and in 39 RA patients at visit 2 (6 weeks).

Figure 2.

Figure 2A: The change in mg 7-OH-MTX / g creatinine (Δ 7-OH-MTX) (visit 3 minus visit 2) on the y axis is plotted versus the change in mg MTX / g creatinine (Δ MTX) (visit 3 minus visit 2) on the x axis, $r = 0.71$ ($n = 35$).

Figure 2B: The change in the percent of the MTX dose excreted as 7-OH-MTX (Δ % of dose) (visit 3 minus visit 2) on the y axis is plotted versus the change in the average mg of the MTX dose retained after 24 hours / kg body weight (Δ μ g MTX / kg) (visit 3 minus visit 2) on the x axis, $r = -0.66$ ($n = 35$).

Table 1
Joint Improvements in Patients is Dependent upon Mean % of MTX Dose Excreted as 7-OH-MTX ^{1, 2}

Category of Joint Improvement	N	Mean % of MTX Dose Excreted as 7-OH-MTX at Visits 2 and 3 (S.D.)
Marked Improvement	9	1.95 (1.48) ³
Moderate Improvement	18	3.15 (1.30)
No Change	8	3.29 (1.34)

¹ANOVA, $F = 5.4$, $p \cong 0.01$, data log transformed before analysis

²Marked improvement: greater than 50% decrease in swelling (SW) and pain/tenderness indices at both visit 2 (6 weeks) and visit 3 (7 weeks); no change: less than 30% change in SW and PT indices at both visit 2 and 3; Moderate improvement: all other patients. No patients were in the category of worsening.

³Significantly less than the other groups, (ANOVA, then Scheffe's test $p < 0.05$).