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Pharmacokinetics, pharmacodynamics, and toxicities of methotrexate in healthy and collagen-induced arthritic rats

Dong-Yang Liu^a, Hoi-Kei Lon^a, Yan-Lin Wang^a, Debra C. DuBois^b, Richard R. Almon^b, and William J. Jusko^{a,*}

^aDepartment of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, Buffalo, NY 14214, USA

^bDepartment of Biological Sciences, University at Buffalo, Buffalo, NY 14260, USA

Abstract

Methotrexate (MTX) is an anchor drug used to treat rheumatoid arthritis (RA), but responsiveness is variable in effectiveness and toxicity. Methotrexate and its polyglutamate conjugates (MTXPG_n) in red blood cells (RBC) have been associated with patient response. In the current study, 13 collagen-induced arthritic (CIA) rats and 12 healthy rats were given subcutaneous doses of either saline or 0.3 or 1.5 mg/kg per 2 days of MTX from day 21 to 43 post-induction. Blood samples were obtained at various times to measure MTX in plasma, and MTX and MTXPG_n in RBC. Effects on disease progression were indicated by body weight and paw size. After multiple-doses, RBC MTX reached steady-state (82.4 nM) within 4 days. The MTXPG₂ and MTXPG₃ in RBC kept increasing until the end of the study attaining 12.5 and 17.7 nM. Significant weight loss was observed after dosing of 1.5 mg/kg/2 days, whereas moderate effectiveness was observed after dosing of 0.3 mg/kg/2 days. A pharmacokinetic/ pharmacodynamic/disease (PK/PD/DIS) model with indirect mechanisms and transduction components incorporating plasma MTX, RBC MTX, and RBC MTXPG_n concentrations, and paw size was developed using naïve data pooling and ADAPT 5. The PK/PD in CIA rats dosed at 0.3 mg/kg/2 days were captured well by our proposed model. MTX showed modest ($I_{max_d} = 0.16$) but sensitive ($IC_{50_d} = 0.712$ nM) effectiveness on paw edema. The higher dose produced toxicity. The proposed model offers improved understanding of MTX effects on rheumatoid arthritis.

Keywords

Methotrexate; rheumatoid arthritis; pharmacokinetics; pharmacodynamics; disease progression

Introduction

Rheumatoid arthritis (RA) is a systemic, autoimmune, and acute-on-chronic inflammatory disorder that primarily targets synovial joints [1]. It significantly impacts quality of life and produces joint pain, stiffness, and swelling due to synovial inflammation and effusion. In severe cases, RA leads to erosions of the inflamed sites, joint deformity, disability, and even reduced life expectancy [2]. It affects about 1.5 million US adults [3]. To treat RA, four major types of drugs are available: non-steroid anti-inflammatory drugs (NSAID), disease-modifying anti-rheumatic drugs (DMARD), biologic agents, and corticosteroids. Methotrexate (MTX) is used most frequently worldwide. It affects several targets, such as

*To whom correspondence should be addressed: Corresponding Author: Dr. William J. Jusko, Department of Pharmaceutical Sciences, 404 Kapoor Hall, State University of New York at Buffalo, Buffalo, NY 14214, Phone: 716-645-2855, FAX: 716-829-6569, wjjusko@buffalo.edu.

apoptosis of activated lymphocytes, and adenosine and cytokine modulation [4, 5]. Such alterations can reduce joint inflammation and bone destruction, two major RA symptoms [6, 7]. The effectiveness of MTX in RA varies from 46–65% with response as defined by American College of Rheumatology (ACR) 20 criteria [8]. It is the anchor drug among the DMARD and is accepted internationally as the first choice in management of RA [9]. In routine clinical care of patients with RA, a comparative analysis in Finland and the United States reported that the ‘early’ use of MTX (within 5 years after disease onset) increased from less than 5% in Finland and less than 25% in Nashville (TN, USA) in 1980 to more than 90% in 2004 in both countries. This was associated with substantially improved outcomes [10].

Although low-dose MTX (7.5–30 mg/week) has been used to treat RA for 30 years, its therapeutic strategy is trial-and-error partly due to an unclear relationship between plasma MTX concentrations and response [11]. There is high inter-individual variability (IIV) in PK and PD for unknown reasons, which leads to unpredictable responses and adverse events [12]. Recently, PK profiles and PD mechanisms of MTX were utilized to explore reasons for high IIV. After subcutaneous (SC) injection, MTX is absorbed rapidly in humans and rats with average bioavailability of 70–80% [13]. After entering blood, MTX is transported into red blood cells (RBC), bone marrow myeloid precursors, white blood cells, hepatocytes, fibroblasts, and synoviocytes, mainly via the reduced folate carrier (RFC) [14]. Once inside cells, MTX is further metabolized to conjugates by folylpolyglutamate synthetase (FPGS) forming up to 6 glutamates (MTXPG₂ – MTXPG₇) [15]. Gamma glutamyl hydrolase (GGH) removes terminal MTX glutamate to form MTX conjugates with fewer glutamates and finally returns MTXPG_n to its monoglutamate form (MTX), which is rapidly transported out of cells by multidrug resistance-associated proteins. Although the mechanism of action is uncertain [16], intracellular MTXPG_n may be a necessary mediator of anti-inflammatory effects [17] and was reported to be associated with MTX effectiveness [18–21]. Therefore, erythrocyte MTXPG_n [22, 23] and polymorphism of those proteins transporting and forming intracellular MTXPG_n [18, 24] were studied to identify responders in RA patients treated with MTX. However, genotypes of those transporters and enzymes showed inconclusive prediction performance. Non-genetic factors such as age, dosage, and renal function, were not considered [22, 25]. Theoretically, MTXPG_n in RBC can relate to responses directly. Therefore, it is possible that MTXPG_n concentrations in RBC may better predict therapeutic response.

The dynamic effects of MTX mediated by MTXPG_n on RA disease progression and toxicology have been incompletely assessed. Use of PK/PD/Disease (PK/PD/DIS) modeling can quantitatively interpret disease progression and assess drug effects in a mechanistic manner [26]. Several physiologically-based PK, multiple-compartment PK, and PK/PD/toxicity models were utilized to describe the kinetics of MTX and MTXPG_n in plasma, tissues [27], and selected cells [28] to seek relationships between PK and effectiveness and toxicity [29, 30]. However, none of these models related MTX effectiveness to intracellular MTXPG_n. The collagen-induced arthritis (CIA) model in rats is an animal disease model which closely reflects major aspects of human RA such as chronic inflammation, involvement of both B and T-cells, and absence of bacteria and mycobacterium components [31, 32]. We utilized the CIA rat model to investigate the effects of MTX on disease progression and toxicology and developed a mechanistic model incorporating RBC MTXPG_n.

Materials and Methods

Drugs and Compounds

Methotrexate (MTX) was purchased from Sigma Inc. (Thousand Oaks, CA). The 4-amino-10-methylpteroylidiglutamic acid (MTXPG₂), 4-amino-10-methylpteroyltriglutamic acid (MTXPG₃), 4-amino-10-methylpteroyltetraglutamic acid (MTXPG₄), 4-amino-10-methylpteroylpentaglutamic acid (MTXPG₅), 4-amino-10-methylpteroylhexaglutamic acid (MTXPG₆), and 4-amino-10-methylpteroylheptaglutamic acid (MTXPG₇) were obtained from Schircks Lab (Jona, Switzerland). Methotrexate-methyl-D₃ (MTX-D₃) was acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Dithiothreitol (DTT), citric acid, and ascorbic acid were obtained from Sigma-Aldrich Co. (St. Louis, MO).

Animals

Thirty-eight male Lewis rats, aged 6 – 9 weeks, were purchased from Harlan (Indianapolis, IN), weight-matched to approximately 150 g. Animals were housed individually in the University Laboratory Animal Facility and acclimatized for 1 week under constant temperature (22°C), humidity (72%), and 12-h light/12-h dark cycle. Rats had free access to rat chow and water. All protocols followed the Principles of Laboratory Animal Care (Institute of Laboratory Animal Resources, 1996) and were approved by the University at Buffalo Institutional Animal Care and Use Committee.

Induction of Collagen-induced Arthritis

The induction of collagen-induced arthritis in Lewis rats followed protocols and reagents supplied by Chondrex, Inc. (Redmond, WA). Porcine collagen type II (2 mg/mL) in 0.05 M acetic acid was emulsified with incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) following procedures described in our earlier study [33].

Experimental Design

Healthy and CIA rats were utilized. In order to minimize stress to animals in the PD study, a separate group of rats were used for serial blood sampling for PK analysis. Thirty-six rats were induced by collagen injection and boosted 7 days later. Two healthy rats and additional induced rats without arthritis (paw size increases less than 15% in either paw on day 20, n=12) were randomly divided into 2 groups. Groups received SC injections of 0.3 mg/kg every two days (PKL group, n=6), or 1.5 mg/kg every 2 days (PKH group, n=6) for PK studies from day 21 (peak of disease progression) to day 43. MTX was dissolved in saline adjusted to pH 8.6. After evaluation of paw edema induction on day 20, 13 CIA rats with paw size increase of at least 50% in one or two paws were selected and randomly assigned to three groups for PD study. Animals received SC injections of saline solution (pH 8.6) (PDC group, n=4), 0.3 mg/kg (PDL group, n=4), or 1.5 mg/kg (PDH group, n=5) of MTX every two days from days 21 to 43. On day 46, PKL and PDL groups continued to be dosed with 0.3 mg/kg. In PKL and PKH groups, blood samples were collected from the saphenous vein using EDTA-coated capillary tubes and transferred to amber tubes on ice. Blood was obtained for plasma MTX and erythrocyte MTXPG_n concentrations at 0.5, 1, 4, and 10 hr on day 1 of drug treatment; before dosing every two days during day 21 through 41; and 0.5, 1, 3, 6, 10, and 24 hr post-dosing on day 46. On day 46, the same blood sampling scheme was applied to the PDL group. RBC numbers were determined using a Cell-Dyn Emerald (Abbott Laboratories, Abbott Park, IL). Remaining blood (100 µL) was centrifuged at 2000 × g for 10 min at 4°C. The plasma fraction was immediately transferred into a polyethylene tube on ice and stored frozen at –80°C until assay for MTX. The RBC were washed with 3-fold volumes of ice-cold saline twice. Packed RBC were mixed with 50 µL of saline and stored frozen at –80°C until assayed for MTX and MTXPG_n.

Measurement of Edema and Body Weight

Edema was indicated by size of the rat hind paws [33]. Two cross-sectional areas were determined with digital calipers (VWR Scientific, Rochester, NY), one on the rat forefoot (paw) and the other at the ankle. Two measurements were made on each section, perpendicular to each other, to define the length and height of the ellipse from which the area was determined. Measurements were made side-to-side and top-to-bottom across the paw at the base of the last food pad. Ankle measurements were made side-to-side (length a) and front-to-back (length b) at a 45° angle across the ankle. The area contained in the ellipse is: $\text{Area} = \pi \cdot a/2 \cdot b/2$. Edema was indicated by the sum of paw and ankle area measures for each hind foot. Paw size measurements and body weights were obtained for PDC, PDL, and PDH groups before induction (0 day) and post-induction on 3, 7, 12, 15, 17, 19, 21, 22, 23, 25, 27, 29, 31, 33, 35, 37, 41, and 43 days.

Bioanalytical Methodology

Plasma MTX, RBC MTX, and RBC MTXPG₂₋₇ concentrations were measured using a validated HPLC-MS/MS method. Around 100 μL of plasma or suspended RBC was mixed with 25 μL of perchloric acid followed by addition of 400 μL of internal standard solution containing 2.5 nM of MTX-D₃, 5% of anti-oxidation buffer (0.5% dithiothreitol (DTT) and 10% of both citric acid and ascorbic acid) in 100 mM ammonium acetate (pH 6.8). The mixed solution was vortexed for 1 min followed by centrifugation at $13000 \times g$ for 10 min at 4°C. The supernatant was loaded on an Oasis HLB cartridge (1 cc, Waters Co., Milford, MA) equilibrated with 1.0 mL of methanol followed by 0.8 mL of 100 mM ammonium acetate buffer (pH 6.8) containing 5% of anti-oxidation buffer (buffer A). The loaded cartridge was washed with 0.8 mL of buffer A followed by elution with 0.8 mL of methanol/water (9/1, v/v). Eluted solutions were collected in a drying cartridge and evaporated by nitrogen gas at 35°C. Dried residue was reconstituted with acetonitrile/water (1/9, v/v) containing 5% of anti-oxidation buffer and stored at 8°C until analysis. Reconstitution solutions were separated on the Symmetry Shield RP₈ (2.1×50 mm, 3.5 μm , Waters Co.) kept at 35°C using a gradient flow of mobile phase consisting of buffer B (water / acetonitrile = 95/5 v/v, containing 0.1% formic acid) and buffer C (acetonitrile / water = 95/5 v/v, containing 0.1% formic acid). The extracted samples were analyzed using a SIL-20A high liquid performance chromatography system (Shimadzu Co., Kyoto, JP), including autosampler, binary pumps, column oven, and controller. The analytes were determined by API 3000 tandem mass spectrometry (Applied Biosystems, Ontario, Canada). The MTXPG_n were determined by multiple reaction monitoring mode with positive ionization using MTX-D₃ as internal standard. The LLOQ were: MTX in plasma (20 nM), MTX and MTXPG₂₋₄ in RBC (2 nM), MTXPG₅ in RBC (5 nM), and MTXPG₆₋₇ in RBC (10 nM).

Validation studies assessed the intra- and inter-run accuracy and precision, stability of RBC samples at room temperature for 2 hr and after 2 cycles of freeze-thaw, and stability of reconstitution samples at 8°C for 24 hr, which met the FDA bioassay guidance [34]. No significant matrix effects were seen. Results were normalized to a RBC count of 8×10^{12} cells to offset variation in RBC counts between individuals and disease periods [35].

PK/PD/DIS Model

A two-compartment model (2CM) with an injection site absorption compartment was used for MTX. An additional three-compartment model with RBC volume (V_3) was used to describe the transformation from MTX to MTXPG₂ and MTXPG₃ in RBC (Figure 1). The MTX in plasma can enter RBC (CL_{pr}) to form MTXPG₂ (CL_{I2}) and MTXPG₃ (CL_{I3}), which can be metabolized back to MTX followed by return to plasma (CL_{rp}). Because

MTXPG_n were only observed in RBC, the rat RBC volume, V₃, was used. The model equations and initial conditions are

$$dA_a/dt = -k_a \times A_a \quad A_{a(0)} = \text{Dose} \quad (1)$$

$$dA_1/dt = k_a \times A_a - CL_e \times A_1/V_1 - CL_d \times (A_1/V_1 - A_2/V_2) - CL_{pr} \times A_1/V_1 + CL_{rp} \times A_{RBC,MTX}/V_3 \quad A_{1(0)} = 0 \quad (2)$$

$$dA_2/dt = CL_d \times (A_1/V_1 - A_2/V_2) \quad A_{2(0)} = 0 \quad (3)$$

$$dA_{RBC,MTX}/dt = CL_{pr} \times A_1/V_1 - CL_{rp} \times A_{RBC,MTX}/V_3 + CL_{12} \times (A_{RBC,MTXPG_2} - A_{RBC,MTX})/V_3 + CL_{13} \times (A_{RBC,MTXPG_3} - A_{RBC,MTX})/V_3 \quad A_{RBC,MTX(0)} = 0 \quad (4)$$

$$dA_{RBC,MTXPG_2}/dt = CL_{12} \times (A_{RBC,MTX} - A_{RBC,MTXPG_2})/V_3 \quad A_{RBC,MTXPG_2(0)} = 0 \quad (5)$$

$$dA_{RBC,MTXPG_3}/dt = CL_{13} \times (A_{RBC,MTX} - A_{RBC,MTXPG_3})/V_3 \quad A_{RBC,MTXPG_3(0)} = 0 \quad (6)$$

where A_a , A_1 , and A_2 are amounts of MTX in the drug absorption, central (V_1) and peripheral (V_2) compartments, $A_{RBC,MTX}$, $A_{RBC,MTXPG_2}$, and $A_{RBC,MTXPG_3}$ are amounts of MTX, MTXPG₂, and MTXPG₃ in erythrocytes, CL_e is elimination clearance of MTX, CL_d is the distribution clearance between V_1 and V_2 , and k_a is the absorption rate constant. In order to accurately estimate distribution and elimination parameters without interference by absorption processes after SC injection, plasma MTX concentration-time data after IV injection was adapted from literature [36]. In this study, 0.1, 0.5, and 2.5 mg/kg of MTX was given to fasted rats (n=5) and blood samples collected over 8 hours. Mean concentrations of plasma MTX from the three dose groups were digitized to allow simultaneous estimation of PK parameters with our present data.

A transduction-based feedback model was used to describe disease progression and drug effects (Fig. 1) [37]. The model equations are

$$dT_1/dt = k_t \times (Paw_0 \times k_{dis} - T_1) \quad (7)$$

$$dT_{n+1}/dt = k_t \times (T_n - T_{n+1}), n=1 \sim 18, 21 \sim 25 \quad (8)$$

$$dT_{21}/dt = k_t \times (T_{19} - T_{21}) \quad (9)$$

$$\frac{dPaw}{dt} = k_{growth} + k_{in} \times T_{19} \times \left(1 - \frac{I_{max_d} \times C_{RBC,MTXPG_3}}{IC_{50_d} + C_{RBC,MTXPG_3}}\right) \times \left(1 - \frac{E_{max_r} \times (T_{25} - Paw_0)}{EC_{50_r} + (T_{25} - Paw_0)}\right) - k_{in} \times Paw \quad (10)$$

where T_1 , T_2 , ..., T_n (n=25) are paw sizes (Paw) in transduction compartments, k_t is a transduction rate constant, k_{dis} is the ratio of maximum paw size to Paw_0 at disease steady-state without remission and drug effects, I_{max_d} and IC_{50_d} are maximum effect of MTX on paw size and RBC MTXPG₃ concentration at 50% of I_{max_d} , the E_{max_r} , EC_{50_r} are maximum effects of natural remission and paw size difference at 50% of E_{max_r} , k_{growth} is the natural growth rate constant of paw size in healthy rats, and k_{in} is the transduction rate constant

between compartment T_{19} and Paw . The initial values for all transit steps, including T_1 , T_2 , $\dots$, T_n , and Paw size (Paw_0), were set as Paw_0 .

Since no difference was observed in PK profiles between healthy and CIA rats, the two groups shared the same PK parameters. The PK and PD/DIS data were naïvely pooled firstly followed by sequential model fitting using the maximum likelihood algorithm in ADAPT 5 (Biomedical Simulations Resource, Los Angeles, CA). The variance model used in both sequential fittings is $V_i = (\sigma_1 + \sigma_2 \times Y_i)^2$, where V_i is the variance of the i th data point, σ_1 and σ_2 are variance model parameters, and Y_i is the i th model prediction. All fitting procedures utilized Windows Vista with the Intel® Visual Fortran Compiler (Version 11.0). Graphical diagnostic analyses were performed using S-Plus (TIBCO Spotfire, Somerville, MA) software.

Results

Toxicology

In the CIA rat study, 36 rats were induced with porcine collagen and boosted 7 days later. Thirteen of the 36 induced rats developed arthritis and were given 0, 0.3, and 1.5 mg/kg/2 days of MTX. Ten of 36 induced rats without arthritis and two additional induction-free rats were given 0.3 and 1.5 mg/kg/2 days of MTX. Body weights (Figure 2) of healthy and CIA rats receiving 1.5 mg/kg/2 days were stable over the first 8 days after dosing and then lost weight. All showed nose bleeding, diarrhea, or rapid body weight loss by day 35, consistent with MTX-induced toxicity. Therefore, CIA rats in the PKH and PDH groups were sacrificed before or on day 35. Paw size time curves of rats in the high dose group are shown in Figure 3. Body weights (Figure 2) of rats receiving 0.3 mg/kg/2 days increased faster than the control group, indicating no MTX-induced toxicity in this group. Therefore, the low dose group was utilized for modeling, whereas the high dose data were not used as being confounded by MTX toxicity.

Pharmacokinetics

Short-term and trough concentration - time curves of MTX, MTXPG₂, and MTXPG₃ in healthy and CIA rats are presented in Figure 4. After SC injection, plasma MTX concentrations reached C_{max} within 0.5 hour. The C_{max} was 3.5-times greater in the high dose group than in low dose group. Thereafter, MTX concentrations decreased biexponentially. The terminal phase concentrations were similar to those after IV dosing [36]. Plasma MTX and RBC MTX, MTXPG₂, and MTXPG₃ concentrations were comparable between healthy and CIA rats at steady-state (Figure 4). For the PKL group, RBC MTX concentrations reached steady-state on day 4 whereas RBC MTXPG₂ and MTXPG₃ concentrations kept increasing to the end of the study. After multiple-dosing of 0.3 mg/kg/2 days of MTX, mean trough concentrations of RBC attained 82.4 nM for MTX, 12.5 nM for MTXPG₂, and 17.7 nM for MTXPG₃ after 23 days of dosing (Figure 4). The RBC MTX trough concentrations are lower in the high dose group than in the low dose group except for the first dosing and even decreased after multiple-dosing. The RBC MTXPG₂ and MTXPG₃ concentrations in the high dose group are slightly higher than or comparable to those in the low dose group.

Since MTX-induced toxicity in the high dose group was inexplicably accompanied by decreased RBC MTX trough concentrations, these data were not used in modeling. Only the low dose data from healthy and CIA rats were utilized. The 2CM with the k_a input well captured MTX PK profiles. A physiological RBC volume was employed for all RBC concentrations using a value of 0.024 L/kg from healthy rats [38]. Biotransformation clearances were added (CL_{12} and CL_{13}) along with MTX influx clearance of CL_{pr} and efflux

clearance of CL_{rp} . Limited post-dose sampling did not support PK estimates of absorption and distribution phases. Therefore, we digitized literature data for model support. The first-order absorption rate constant (k_a) was fixed to 3.69 hr^{-1} scaled with an exponential constant of -0.031 as calculated from monkey ($k_a = 2.84 \text{ hr}^{-1}$, body weight = 8.8 kg) [39] and human ($k_a = 0.36 \text{ hr}^{-1}$, body weight = 76 kg) [40] SC injection data.

Plasma MTX concentrations (Figure 4) were captured well for the present study and all three dose groups digitized from the literature except for the terminal phase in the high dose group (2.5 mg/kg), where a slight systematic underestimation was found. This deviation was not relevant to our PK/PD/DIS model fitted with low dose data.

The final estimates of PK parameters are listed in Table 1. The systemic clearance (CL_e) was 0.409 L/hr/kg . The V_I (0.174 L/kg) is higher than plasma volume (0.0312 L/kg). The V_2 (0.326 L/kg) is comparable to intracellular fluid volume (0.371 L/kg). The influx and efflux rate constants are very small (0.00564 and 0.012 hr^{-1}) which suggests that MTX takes days to enter and exit erythrocytes. The metabolic rate constants are even lower, consistent with the slow accumulation of the metabolites. Precision of all parameter estimations in this PD/DIS model are less than 40%, reflective of reliable estimates.

Pharmacodynamics and Disease Progression

The time course of paw sizes in CIA rats are displayed in Figure 3. The control group exhibited typical disease onset followed by a remission phase. After low doses (0.3 mg/kg/2 days) of MTX, paw size rapidly decreased in the first 4 days. This group also showed greater increases in body weights (Figure 2). Both profiles suggest that low dose MTX is effective in CIA rats. The final estimated PK/PD/DIS parameters are listed in Table 2. The k_{growth} was fixed as $0.0130 \text{ mm}^2/\text{hr}$ as reported [33] for similar rats. The logistic growth rate constant (k_{dis}) was 19.1 and maximum effect of natural remission (E_{max_r}) was 0.939, which accounts for paw sizes at the remission phase. The paw size at 50% of maximum remission (EC_{50_r}) was 24.5 mm^2 , which indicates that the feedback inhibition on paw growth occurs after paw sizes reach 24.5 mm^2 above baseline. Maximum effect of MTX on paw size (I_{max_d}) was 0.160 and the RBC MTXPG₃ IC_{50_d} was 0.712 nM . The former value means paw swelling is modestly relieved by MTX with the potential of 16% reduction.

Discussion

We measured RBC MTX polyglutamates and observed three MTX glutamates in rat RBC. The RBC MTX was associated with adverse effects earlier than body weight changes. Based on our understanding of MTX mechanism of action, we constructed a PK/PD/DIS model to relate effects to exposure of MTX metabolites. As *in vitro* studies reported that MTXPG₃ is ten-fold more potent than MTX and MTXPG₂ for inhibition of the major anti-RA target enzyme, 5-aminoimidazole-4-carbox-amide ribonucleotide (AICAR) transformylase [41], MTXPG₃ was utilized in Eq. 10 to inhibit paw size in CIA rats in our PK/PD/DIS model.

MTX can temporarily elevate liver enzymes in 10–43% of RA patients. After four years of MTX treatment, mild and severe fibrosis occurs in 15.3% and 1.3% of patients [42]. Monitoring biochemical factors every 2–4 weeks was recommended in RA patients over the first 4 months followed by monitoring every 8–12 weeks in RA patients taking stable doses of MTX [43]. We observed that trough RBC MTX concentrations were higher in rats in the high dose group than in the low dose group on day 2 whereas it was opposite for the rest of the time. This inverse RBC MTX concentration was observed 4 days earlier than reduced body weight, also an indicator of MTX-induced toxicity in mice [29]. Since intracellular MTX exerts anti-folic acid action and causes side effects thereafter [44], decreased RBC MTX concentrations may predict side effects earlier than those biochemical factors

produced after damage. Concentrations (mean $\pm$ SD) in liver were MTX (440 ± 215 nmol/g), MTXPG₂ (600 ± 35 nmol/g), MTXPG₃ (855 ± 442 nmol/g), MTXPG₄ (234 ± 132 nmol/g), and total MTXPG_n (2128 ± 330 nmol/g) in rats with MTX-induced toxicity. After single doses of 0.2 mg/kg of MTX in CIA rats and multiple-dosing of 4 mg/kg/week of MTX in monkeys, total concentration of MTX and MTXPG_n in liver were around 1000 and 2800 nmol/g tissue [45, 46], which are comparable to our values.

MTX was absorbed quickly after SC injection with a scaled k_a of 3.69 hr^{-1} in rats. After entering blood, plasma MTX concentrations decreased biexponentially. The sum of V_1 of 0.174 and V_2 of 0.326 L/kg are comparable to body water volume [38], which suggests moderate distribution of the drug. Plasma MTX concentrations for the 3 dose levels merged in the terminal phase [36], which suggested saturable nonlinear distribution [47]. This might be due to active transport for influx and efflux of MTX by reduced folate carrier (RFC), ABCB1, and ABCC1 transporters on cell membranes [48], which are saturable, pH- and energy-dependent [49]. To capture the saturable distribution, we tested nonlinear influx and efflux clearance in our MTX PK model, which did not yield precise estimates. Since mature erythrocytes lose their nucleus in the last stage of erythropoiesis, it cannot supply energy for ATP-dependent transporters on RBC membranes [50]. Therefore, MTX may be taken up during formation of erythrocytes and persist throughout the RBC lifespan. This is the same fate as folate in erythrocytes [51]. However, adding a time-dependent volume of RBC in our model did not work. The estimated long turnover time of influx, efflux, and transformation of RBC MTX in our model includes rat RBC lifespans of 55 days [52]. The estimated CL_e is comparable to glomerular filtration rate in healthy rats (0.314 L/hr/kg) [38] considering 70–80% bioavailability after SC injection [13] and predominantly renal excretion [53]. After 23 days of dosing, RBC MTX (82.4 nM), MTXPG₂ (12.5 nM), and MTXPG₃ (17.7 nM) concentrations were estimated well in our model. These concentrations are comparable to corresponding values (30, 30, and 60 nM) in RA patients [22]. The RBC MTX reached steady-state quickly, whereas MTXPG₂ and MTXPG₃ kept increasing. The RBC MTXPG₃ concentrations are slightly higher than MTXPG₂, which was also observed in patients with RA [54]. In humans, RBC MTXPG₃ concentrations are even higher than RBC MTX, which suggests specific binding for RBC MTXPG₃ or different conjugation and degradation rates. To model this special profile using our limited data, different metabolic rates were tested to capture the various concentration-time curves. Using different metabolic rate constants for FPGS and GGH to describe the conversions from MTX to MTXPG₂ and from MTXPG₂ to MTXPG₃ was found to overestimate MTXPG₂ concentrations. Studies of the intracellular transformation and distribution processes for MTX are warranted.

In human RA, a latent phase of years with only immune responses evident is followed by the emergence of several pathological inflammatory symptoms such as joint destruction, infections, or osteoporosis [2]. Many cells (e.g. T cells, B cells, and macrophages) and their products (e.g. cytokines, rheumatoid factors, and chemokines) are involved. This disease progression can be mimicked in a short time frame by the CIA model in rats [31, 32], in whom four phases of disease progression were observed: a long delay to develop rheumatoid arthritis after induction, rapid disease worsening with a sharp increase of paw size, a natural remission, and new disease steady-state caused partly by irreversible damage. Gamma functions and an empirical biexponential equation can be utilized to capture the long delay of inflammatory disease. However, in comparison of transduction models, they are less flexible when dose-response relationships, receptor dynamics, and efficiency of the transduction process are considered [37]. The present number of transit steps provided optimal fittings of disease progression. In order to capture all four phases in a natural way, a transduction-based feedback model with logistic growth rate (Figure 1) was applied. Natural growth (k_{growth}) in healthy rats was also incorporated. Briefly, 19 transduction steps (T_1 – T_{19}) were selected to account for the time delay to sharp increase of paw size and 5 more

transduction steps (T_{21} - T_{25}) were used to account for the time delay of natural remission after disease onset. A different transduction rate (from T_{19} to paw size, k_{in}) was needed to fit paw sizes. A logistic growth rate constant (k_{dis}) was used to capture rapid increases in paw size followed by a new disease steady-state. Without the natural remission feedback, paw size was estimated to increase by 19.1-fold above baseline, which is similar to our previous study [37]. A nonlinear negative feedback linking the paw size difference between baseline and disease was utilized to capture the natural remission. A larger increase of paw size will produce greater feedback, but the negative feedback function cannot obviate the entire paw swelling (0.939 of E_{max_r}) due to irreversible damage of the paw. MTX dosing with 0.33–2.5 mg/kg on alternate days was reported to improve arthritic symptoms by 70% in CIA rats [55, 56]. In those studies, MTX was given shortly after disease onset. A moderate but obvious effectiveness ($I_{max} = 0.16$) was observed in the present study. Lesser effects were caused by the later treatment in the present study than in reported studies because timing of drug administration appears to be of considerable importance [37, 57]. The IC_{50_d} of RBC MTXPG₃ was estimated to be 0.712 nM, which means arthritis is sensitive to MTX.

A sensitivity analysis was performed to assess the reliability of IC_{50_d} and I_{max_d} by assessing differences in fittings with higher and lower values. In accordance with the high CV%, the fittings are insensitive to a 100-fold range of IC_{50_d} values. In contrast, the more critical parameter, I_{max_d} , was reliably estimated as indicated by the low CV% (49.7%) and divergence in fittings with 5-fold changes in assigned I_{max_d} values.

Conclusions

The PK/PD and toxicity of MTX in CIA rats were investigated. Multiple doses of 1.5 mg/kg/2 days of MTX produced, significant toxicity. These animals exhibited lower RBC MTX concentrations than after dosing of 0.3 mg/kg/2 days. After multiple doses of 0.3 mg/kg/2 days of MTX in CIA rats, RBC MTX reached steady-state (82.4 nM) within 4 days. The RBC MTXPG₂ and MTXPG₃ kept increasing to the end of the study. Our PK/PD/DIS model incorporating plasma MTX, RBC MTX and MTXPG_n, and paw size captured the PK/PD profiles of MTX and disease progression. MTX showed moderate ($I_{max_d} = 0.16$) but sensitive ($IC_{50_d} = 0.712$ nM) effectiveness on paw edema in CIA rats.

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Figure 1. Schematic of the PK/PD/DIS model for the effects of MTX on paw size and disease progression in CIA rats. Tables 1 and 2 provide definitions of parameters.

Figure 2.
Body weight versus time curves for rats in the indicated study groups.

Figure 3.
Observed and model-fitted paw size versus time curves for CIA rats in three study groups.

Figure 4.

Observed (symbols) and model-fitted (lines) plasma MTX, RBC MTX, MTXPG₂, and MTXPG₃ concentration versus time curves of rats after IV single injection (from literature) of 0.1 (△), 0.5 (◇), and 2.5 mg/kg (▽), and SC injection of 0.3 (PKL□; PDL■) and 1.5 mg/kg/2 days (PKH○).

Table 1Pharmacokinetic parameters for MTX and MTXPG_n in healthy and CIA rats

Parameter (units)	Definition	Estimate	CV%
k_a (hr ⁻¹)	First-order absorption rate	3.69	FIXED ^a
CL_e (L/hr/kg)	Systemic clearance	0.409	9.05
V_1 (L/kg)	Volume of central compartment	0.174	16.4
CL_d (L/hr/kg)	Distribution clearance	0.0707	19.8
V_2 (L/kg)	Volume of peripheral compartment	0.326	35.8
CL_{pr} (L/hr/kg)	Influx clearance of MTX from V_1 to RBC	0.000979	19.8
CL_{I2} (L/hr/kg)	Metabolic clearance of MTX to MTXPG ₂ in RBC	0.00000491	7.04
CL_{I3} (L/hr/kg)	Metabolic clearance of MTX to MTXPG ₃ in RBC	0.00000958	6.18
CL_{rp} (L/hr/kg)	Efflux clearance of MTX from RBC to V_1	0.000289	18.5
V_3 (L/kg)	Volume of RBC	0.024	Fixed ^b
k_{pr} (hr ⁻¹)	Influx rate constant of MTX from V_1 to RBC	0.00564	<i>c</i>
k_{I2} (hr ⁻¹)	Metabolic rate constant of MTX to MTXPG ₂ in RBC	0.00020	<i>c</i>
k_{23} (hr ⁻¹)	Metabolic rate constant of MTX to MTXPG ₃ in RBC	0.0004	<i>c</i>
k_{rp} (hr ⁻¹)	Efflux rate constant of MTX from RBC to V_1	0.0120	<i>c</i>

^aParameter fixed to value scaled from monkeys and human data in literature^bParameter fixed to value from healthy rats in literature^cSecondary parameter

Table 2

Pharmacodynamic/disease parameters for MTX in CIA rats

Parameter (units)	Definition	Estimate	CV%
k_{dis}	Ratio of max. paw size to Paw_0 at disease steady-state without remission and drug effects	19.1	154
k_t (hr^{-1})	Transduction rate between $T_1 \sim T_{25}$	0.0432	17.4
Paw_0 (mm^2)	Baseline of paw size	54.2	2.0
k_{in} (hr^{-1})	Transduction rate between T_{19} and Paw	0.00417	77.7
k_{growth} (mm^2/hr)	Natural growth of paw size	0.0130	Fixed ^a
E_{max_r}	Maximum effect of natural remission	0.939	9.85
EC_{50_r} (mm^2)	Paw size for 50% of E_{max_r}	24.5	78.6
I_{max_d}	Maximum effect of MTX on paw size	0.160	49.7
IC_{50_d} (nM)	RBC MTXPG ₃ conc. for 50% of I_{max_d}	0.712	498

^aParameter fixed to values from healthy rats in literature