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Intracellular CD3$^+$ T Lymphocyte Teriflunomide Concentration Is Poorly Correlated with and Has Greater Variability Than Unbound Plasma Teriflunomide Concentration

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ABSTRACT

Leflunomide's active metabolite teriflunomide inhibits dihydro-oroate dehydrogenase, an enzyme essential to proliferation of T lymphocytes. As teriflunomide must reach the target site to have this effect, this study assessed the distribution of teriflunomide into T lymphocytes, as intracellular concentrations may be a superior response biomarker to plasma concentrations. CD3 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used to extract CD3$^+$ T cells from the peripheral blood of patients with rheumatoid arthritis who were taking a stable dose of leflunomide. Unbound plasma and intra-CD3$^+$ T cell teriflunomide concentrations were quantified using liquid chromatography–mass spectrometry. Concentration (log transformed) and partition differences were assessed through paired Student *t* tests. Sixteen patients provided plasma steady-state teriflunomide samples, and eight provided a sample 6–12 weeks later. At time-point one, the geometric mean teriflunomide concentration (range) in CD3$^+$ T cells was 18.12 μg/L (6.15–42.26 μg/L) compared with 69.75 μg/L (32.89–263.1 μg/L) unbound in plasma ($P < 0.001$). The mean partition coefficient (range) for unbound plasma teriflunomide into CD3$^+$ T cells was 0.295 (0.092–0.632), which was significantly different from unity ($P < 0.001$). The median (range) change in teriflunomide concentration between the two time points was 14% (−10% to 40%) in unbound plasma and −29% (−69 to 138%) for CD3$^+$ T cells. Because teriflunomide concentrations in CD3$^+$ T cells were lower and displayed a higher intraindividual variability than the unbound plasma concentrations, its applicability as a therapeutic drug-monitoring marker may be limited.

Introduction

Leflunomide is a disease-modifying antirheumatic drug (DMARD) with comparable efficacy to methotrexate, the gold standard DMARD used in the treatment of rheumatoid arthritis (RA) (Alcorn et al., 2009). The therapeutic effects of leflunomide are mediated via its active metabolite teriflunomide (Breedveld and Dayer, 2000), which at therapeutic doses inhibits the mitochondrial enzyme dihydro-oroate dehydrogenase (DHODH), which is essential for de novo pyrimidine synthesis in activated T lymphocytes (Breedveld and Dayer, 2000). However, response to therapy is variable, and it may take up to 6 months for efficacy to be recognized, possibly due to the long half-life (~15 days) of teriflunomide (Rozman, 2002). Furthermore, 20–40% of patients discontinue therapy due to toxicity (Aletaha et al., 2003; Alcorn et al., 2009). It has been hypothesized that this variability in response is related to the highly variable teriflunomide plasma concentrations, which range from 15 to 98 μg/mL and 3 to 150 μg/mL in individuals taking 10 and 20 mg doses, respectively (Schmidt et al., 2003; Van Roon et al., 2005; Bohanec Grabar, 2009). Although a total teriflunomide plasma concentration–effect relationship has been demonstrated, reported concentration thresholds for efficacy vary, ranging from 16 to 50 μg/mL (Chan et al., 2005; Van Roon et al., 2005; Bohanec Grabar, 2009), and, as such, target concentration interventions (TCI) are not used clinically.

Nonetheless, a superior target metric may enable the TCI strategy to be used to improve response to leflunomide. Because teriflunomide is extensively bound to plasma proteins (>99%), modest variability in the fraction unbound can lead to large variability in unbound teriflunomide concentrations, and unbound concentrations may be more closely related to response (Rakhila et al., 2011). Because the primary site of teriflunomide's therapeutic effect is within T lymphocytes, intracellular concentrations may also be more predictive of response. Given the significant advances in the sensitivity of mass-spectrometry techniques over the past decade, quantification of intracellular concentrations appears feasible. As such, the aims of this study were as follows:

1. Develop a method to quantify teriflunomide concentrations within peripheral blood (PB) CD3$^+$ T lymphocytes (CD3$^+$

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ABBREVIATIONS: BSV, between-subject variability; DHODH, dihydro-oroate dehydrogenase; DMARD, disease-modifying antirheumatic drug; HPLC, high-performance liquid chromatography; ID, identification number; LLOQ, lower limit of quantification; MNC, mononuclear cell; PB, peripheral blood; PBS, phosphate-buffered saline; pHi, intracellular pH; QC, quality control; RA, rheumatoid arthritis; RBC, red blood cell; TCI, target concentration interventions; WSV, within-subject variability.

T cells) and nontarget reference cells [i.e., red blood cells (RBC) and CD3− mononuclear cells (CD3− MNC)].
2. Determine the partition coefficient of teriflunomide into CD3+ T cells and the nontarget cells with respect to unbound plasma teriflunomide concentrations.
3. Determine the between-subject variability (BSV) and within-subject variability (WSV) of teriflunomide concentrations within CD3+ T cells, nontarget cells, and plasma.
4. Determine the BSV and WSV of the partition coefficient of unbound plasma teriflunomide into CD3+ T cells and nontarget cells.

The overall hypothesis was that this information would lead to large-scale studies relating intracellular T lymphocyte teriflunomide concentrations to efficacy and toxicity, and ultimately allow a model-based analysis of the potential for intracellular T lymphocyte teriflunomide concentrations as a TCI.

Materials and Methods

Chemicals and Reagents

Teriflunomide (A77 1726) and D4-teriflunomide (A77 1726-D4; isotopic purity: 99%) were obtained from Toronto Research Chemical (Toronto, Canada) and stored in methanol (analytical grade; Mallinckrodt Chemicals, Phillipsburg, NJ) at −80°C. Water was Milli-RQ grade (EMD Millipore, Billerica, Massachusetts). Acetonitrile (analytical grade), ammonium acetate, zinc sulfate ($ZnSO_4$), disodium phosphate (Na_2HPO_4), and sodium chloride (NaCl) were obtained from Merck (Darmstadt, Germany). Drug-free (blank) human plasma was prepared from PB, collected from healthy volunteers, and stored at −80°C. Lymphoprep was purchased from Axis-Shield (Oslo, Norway), whereas trypan blue solution (0.4%, sterile filtered) was from Sigma-Aldrich (Irvine, Scotland). CD3 MicroBeads human (lyophilized), MS MACS separation columns, and a MiniMACS magnetic separator were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Endotoxin-free 1× phosphate-buffered saline (PBS) and fetal calf serum were obtained from the Media Production Unit (Institute of Medical and Veterinary Science, Adelaide, Australia). Wash buffer was prepared with PBS containing 2% fetal calf serum.

Study Population

Subjects attending the Early Arthritis Clinic at the Royal Adelaide Hospital were eligible for inclusion in this study. All participants were aged ≥18 years with DMARD-naive RA, according to revised American College of Rheumatology (ACR) Criteria (Arnett et al., 1988). RA was treated according to a previously published treat-to-target protocol, in which newly diagnosed patients were initiated on triple therapy (methotrexate, sulfasalazine, and hydroxychloroquine) (Proudman et al., 2007). In those failing to respond to optimal dosing of triple therapy, leflunomide was added. Inclusion criteria for this study included participants on a stable dose of leflunomide for the previous 8 weeks (to ensure steady-state plasma teriflunomide concentrations). Participants who remained on the same leflunomide dose at their next clinic visit (6–12 weeks later) were invited to provide a second blood sample. Patients gave informed written consent for inclusion in the study, and ethics approval was obtained from the Human Research Ethics Committee of the Royal Adelaide Hospital and the University of South Australia.

Sample Collections and Processing

At two consecutive clinical visits (T1 and T2), participants provided predose PB samples (~40 mL), which were collected in heparinized vacutainers. A fraction of the PB sample (2 mL) was centrifuged at 10,000 rpm for 15 minutes at room temperature, after which plasma was removed and stored at −80°C for later assay. The volume of remaining PB was recorded and diluted 1:1 in sterile PBS. This was then layered on a Lymphoprep density gradient and centrifuged at 1717 rpm for 20 minutes at room temperature. The MNC and RBC layers were collected and stored on ice.

The RBC interface was washed with PBS and centrifuged at 3135 rpm for 10 minutes at 4°C; the supernatant was aspirated; and RBC were stored at −80°C for later assay. The MNC interface was washed with wash buffer and centrifuged at 1200 rpm for 10 minutes at 4°C, after which the supernatant was aspirated and the number of MNCs was counted.

CD3+ T cells were isolated from the harvested MNC by positive magnetic selection using CD3 MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions. Briefly, the harvested MNCs were resuspended in wash buffer, filtered through a nylon cell strainer (40 μm pore size; BD Biosciences, Franklin Lakes, New Jersey), and centrifuged at 1200 rpm for 10 minutes at 4°C. The supernatant was aspirated, and the pellet was resuspended in 80 μL wash buffer plus 20 μL CD3 MicroBeads per 10^7 total cells. Cells were mixed well and incubated for 15 minutes at 4°C, and then washed with 1–2 mL wash buffer per 10^7 total cells and centrifuged at 1200 rpm for 10 minutes at 4°C. The supernatant was aspirated, and the pellet was resuspended in 500 μL wash buffer. Within a magnetic field, the cell suspension was applied to MS MACS separation column and rinsed with 3× 500 μL wash buffer. CD3− MNC passed through the column and were collected, counted, centrifuged, and stored in 200 μL water at −80°C for later assay. The separation column was removed from the magnetic field and loaded with 1 mL wash buffer, and isolated CD3+ T cells were collected, counted, centrifuged, and stored in 200 μL water at −80°C.

LCMS-8050 Triple Quadrupole Liquid Chromatograph Mass Spectrometer (LC-MS/MS) Method

Separation and quantification of teriflunomide were performed using a modified method of Rakhila et al. (2011), and used a reversed-phase column [Kinetex 2.6 μm C18, 100A, 50X2.1 mm (Phenomenex, Torrance, California)] with a C18 pre-column cartridge [SecurityGuard Ultra 2.1 mm (Phenomenex)]. Separation of teriflunomide and D4-teriflunomide was achieved using two LC-30AS liquid chromatographs, a SIL-30AC autosampler, a CBM-20A communications bus module, a CTO-20AC column oven, a DGU-20A5R degassing unit (Shimadzu, Nakagyo-ku, Kyoto, Japan), and a reverse-phase gradient method. Mobile phase A was composed of water–acetonitrile–1 M ammonium acetate in water at 95:5:0.05 v/v/v, and mobile phase B was composed of water–acetonitrile–1 M ammonium acetate in water at 5:95:0.05 v/v/v. The flow rate was 0.5 mL/min. Initially, mobile-phase composition was maintained at 15% of mobile phase B from 0 to 0.2 minute, then from 0.2 to 1.0 minute mobile-phase B was increased via a linear gradient to 80%, and maintained at 80% until 1.5 minutes, after which the percentage of mobile phase B was reduced to 15% via a linear gradient from 1.5 to 2.5 minutes, which was maintained until 3.5 minutes, followed by the next injection. Detection occurred with the use of a LCMS-8050 triple quadrupole MS (Shimadzu). Quantitation was achieved in negative ion mode with a dwell time of 147 milliseconds. Q1/Q3 transition was 269.1/160.0 amu and 273.1/164.0 amu for teriflunomide and D4-teriflunomide, respectively. Calibration curves were obtained using a linear regression algorithm using the peak area ratio of teriflunomide to d4-teriflunomide, with a weighting factor of $1/C^2$. Calibration curves were deemed acceptable if the mean accuracy and percentage relative S.D. of the triplicate calibration standards were within ±15%, or within ±20% at the lower limit of quantification (LLOQ).

Plasma Sample Preparation. Unbound teriflunomide was separated from bound using Rapid Equilibrium Dialysis plates, according to the manufacturer's instructions (ThermoFisher, Waltham, MA), in which 400 μL plasma (adjusted to pH 7.4) was dialyzed against isotonic PBS solution (pH 7.4) for 6 hours. Bound and total teriflunomide were diluted 1:200 in PBS, and unbound fraction was neat. Calibration standards were prepared using blank freeze-thawed plasma diluted 1:200 with PBS. To 100 μL standard dilution, 10 μL D4-teriflunomide (100 μg/L) and increasing amounts of teriflunomide (1000 μg/L) were added to prepare solutions containing 0, 5, 10, 15, 20, 40, 100, 200, 300, 400, and 500 μg/L. Samples were made to 160 μL with methanol, to which $ZnSO_4$ (100 μL, 0.2 M) and acetonitrile (100 μL) were added to precipitate proteins. Tubes were capped, vortexed for 10 seconds, and centrifuged at 4000 rpm for 10 minutes at 4°C, and the clear supernatant was removed. Quality controls (QC; 15, 80, and 400 μg/L) and test samples were treated in the same manner, except that methanol was added to the test samples instead of the teriflunomide stock solution. For all samples and calibration standards, 1 μL was injected onto the high-performance liquid chromatography (HPLC) column for analysis. Injection of each standard and QC was made in triplicate.

RBC Sample Preparation

Calibration standards were prepared using blank freeze-thawed packed RBC diluted 1:10 with water. To 100 μL dilution, 10 μL D4-teriflunomide (100 μg/L)

and increasing amounts of teriflunomide (1000 μg/L) were added to prepare solutions containing 0, 5, 10, 15, 20, 40, 100, 200, 300, 400, and 500 μg/L. Samples were made to 160 μL with methanol, to which water (200 μL), $ZnSO_4$ (300 μL, 0.2 M), and acetonitrile (300 μL) were added to precipitate proteins. Tubes were capped, vortexed for 10 seconds, and centrifuged at 4000 rpm for 10 minutes at 4°C, and the clear supernatant was removed. QCs (15, 80, and 400 μg/L) and test samples were treated in the same manner, except that methanol was added to the test samples instead of the teriflunomide stock solution. For analysis, 0.5 μL supernatant was injected onto the HPLC column. Injection of each standard and QC was made in triplicate.

Mononuclear Cell Sample Preparation

Calibration standards were prepared by using 200 μL thawed wash buffer, to which 10 μL D4-teriflunomide (20 μg/L) and increasing amounts of teriflunomide (20 μg/L) were added to prepare solutions containing 0, 5, 10, 20, 25, 50, 100, and 200 pg/sample. Samples were made to 310 μL with methanol, to which 300 μL acetonitrile was added. This solution was then vacuum dried at 1400 rpm and 60°C (Concentrator Plus; Eppendorf, Hamburg, Germany). The dried sample was reconstituted in 55 μL acetonitrile, 30 μL methanol, and 115 μL water, and vortexed for 15 minutes. The sample was then centrifuged at 4000 rpm for 10 minutes at 4°C, and the clear supernatant was removed. QC (20, 100, and 800 pg/sample) and test samples were treated in the same manner, except that methanol was added instead of teriflunomide stock solution, and wash buffer was exchanged for freeze-thawed $CD3^+$ T cells or $CD3^-$ MNC samples. For analysis, 5 μL $CD3^+$ T cell supernatant and 2 μL $CD3^-$ MNC supernatant were injected onto the HPLC column. Each run included a standard curve, and injection of each standard and QC was made in triplicate.

Statistical Analysis

For the present study, intracellular concentrations (pg/10^6 cells) of teriflunomide in $CD3^+$ T cells and $CD3^-$ MNC were transformed into μg/L values, by considering that the volume of 10^6 $CD3^+$ T cells and $CD3^-$ MNC was 1.76×10^{-7} L and 1.87×10^{-7} L, respectively (Chapman et al., 1981; Bazzoli et al., 2011). The partition of teriflunomide into cell matrices was calculated by dividing $CD3^+$ T cell, $CD3^-$ MNC, and RBC teriflunomide concentrations by the unbound plasma teriflunomide concentration. The difference between the geometric means of log-transformed teriflunomide concentrations in $CD3^+$ T cells, $CD3^-$ MNC, RBC, and plasma (unbound and total) between the two visits was assessed via a paired Student *t* test. A paired Student *t* test was also used to compare the difference of geometric means between log-transformed $CD3^+$ T cell teriflunomide concentrations and unbound teriflunomide concentrations at T1. The mean of the partition coefficient of teriflunomide from unbound plasma to $CD3^+$ T cells at T1 was compared against a unity of 1 via a paired Student *t* test.

The R Software Version 3.1.1 (R Core Team, 2015) was used for data manipulation, graphical output, and statistical analysis using the ggplot2, doBy, reshape, and GGally packages (Wickham, 2007, 2009; Højsgaard et al., 2014) (http://cran.r-project.org/web/packages/GGally/index.html). Linear mixed-effect modeling with the lme4 package (http://cran.r-project.org/web/packages/lme4/index.html) was used to assess the between and within individual variance of log-transformed $CD3^+$ T cell, $CD3^-$ MNC, RBC, unbound and total teriflunomide concentrations, and unbound to $CD3^+$ T cell, unbound to $CD3^-$ MNC, and unbound to RBC teriflunomide partition. Subject identification number (ID) was accounted for as a random effect, in which the S.D. of the intercept represents BSV and the S.D. of the residuals represents WSV (Winter, 2013).

Results

Patient Demographics. Seventeen participants were recruited, nine of whom provided a second matched dose steady-state sample. One participant was excluded as the teriflunomide concentration within $CD3^+$ T cells for the first sample was below the LLOQ, and for the second sample the teriflunomide concentrations within $CD3^+$ T cells, $CD3^-$ MNC and RBC, and the total and unbound plasma concentration were below the LLOQ. The characteristics of the remaining 16 participants are described in Table 1.

The median (range) volume of whole blood was 30 mL (17.5–32.5 mL) for the 24 samples. The median (range) number of $CD3^+$ T cells and $CD3^-$ MNC isolated from these samples was 9.30×10^6 cells (2.80×10^6–2.62×10^7 cells) and 5.78×10^7cells (3.00×10^7–8.10×10^7 cells), respectively.

LC-MS/MS Method. The LLOQ for $CD3^+$ T cells, $CD3^-$ MNC, RBC, and the total and unbound plasma teriflunomide concentrations were 10 pg/sample, 10 pg/sample, 100 μg/L, 1 mg/L, and 5 ug/L, respectively. The mean accuracy (percentage relative S.D.) of the triplicate calibration standards of the $CD3^+$ T cell teriflunomide concentration curve at 10, 25, and 200 pg/sample was 97.9% (11.2%), 106.9% (7.2%), and 88.2% (0.65%), respectively. The $CD3^+$ T cell teriflunomide concentration curve extended to the lowest LLOQ (10 pg/sample) of the investigated metrics, and all other curves (i.e., from the different matrices) performed equally well or better.

Intracellular Teriflunomide Concentrations. For the 16 samples at T1, $CD3^+$ T cell, $CD3^-$ MNC, RBC, unbound, and total plasma teriflunomide concentrations had a median (range; %CV) of 19.57 μg/L (6.15–42.26 μg/L; 49.03%), 21.42 μg/L (4.45–41.79 μg/L; 51.50%), 1463.9 μg/L (799.5–5039.4 μg/L; 60.84%), 67.06 μg/L (32.89–263.10 μg/L; 70.05%), and 27.58 mg/L (15.02–115.03 mg/L; 70.03%), respectively (Fig. 1). T1 $CD3^+$ T cell teriflunomide concentrations were significantly lower than unbound plasma teriflunomide concentrations ($P < 0.001$). Geometric mean $CD3^+$ T cell, $CD3^-$ MNC, RBC, unbound, and total teriflunomide concentrations were not significantly different between T1 and T2 ($P > 0.05$).

The $CD3^+$ T cell, $CD3^-$ MNC, RBC, unbound, and total plasma teriflunomide concentrations were described by the linear mixed-effect model represented below, in which subject ID is a random effect representing the BSV, and ε quantifies the unknown random effects (residuals), which in part represent the WSV (Winter, 2013):

TABLE 1

Patient demographics

IQR presents the first and third quartiles.

Characteristics			
Number of participants		16	
T2 samples		8	
		T1	T2
Age (years)	Median (IQR)	61.3 (50.0–67.7)	61.6 (55.6–70.9)
Gender	Male	5	3
	Female	11	5
Leflunomide dose	10 mg daily 2 of 3 days	1	0
	10 mg daily	3	3
	20 mg daily	11	5
	10 and 20 mg alternate days	1	0
MNC ($\times 10^9$ cells/L)	Median (IQR)	1.91 (1.40–2.26)	2.18 (1.62–2.92)

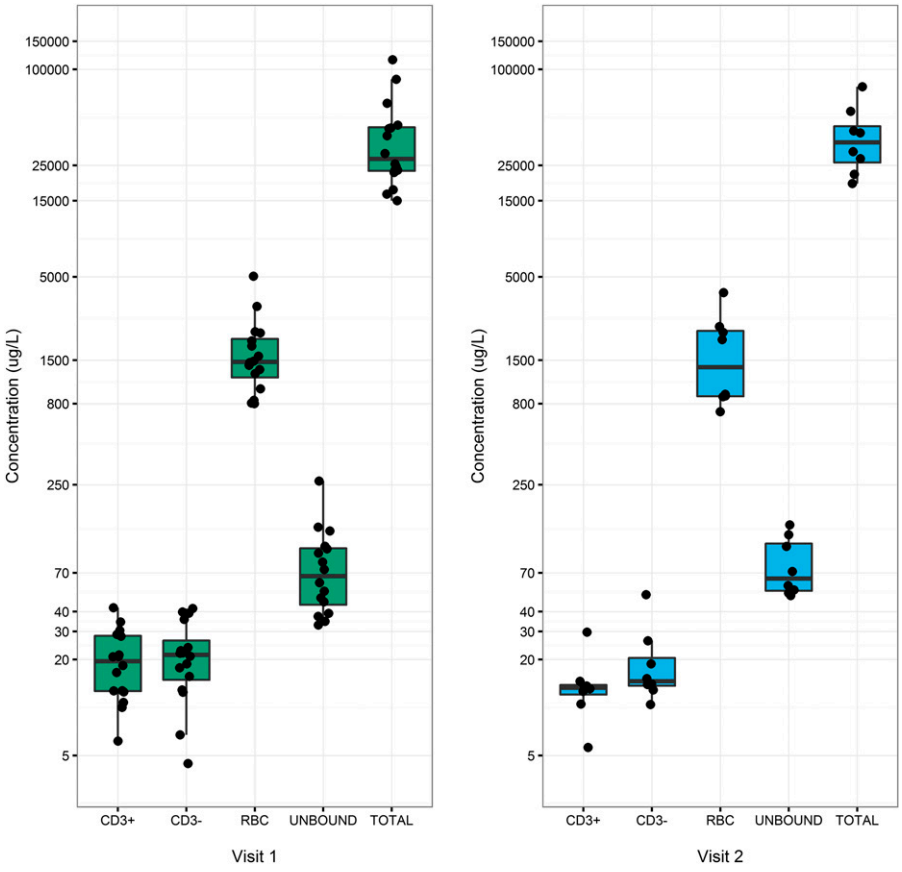

Fig. 1. Box plot of CD3$^+$ T cell, CD3$^-$ MNC, RBC, unbound, and total teriflunomide concentrations at T1 and T2.

$$\log(\text{Concentration metric}) \sim 1 + (1|\text{ID}) + \varepsilon \quad (1)$$

For unbound and total teriflunomide concentrations, the BSV was greater than the WSV (Table 2). For the RBC teriflunomide concentrations, the BSV was comparable to the WSV. For CD3$^+$ T cells and CD3$^-$ MNC teriflunomide concentrations, the BSV had a trend toward being less than the WSV, although there was overlap of the 90% confidence intervals of the S.D. estimates for the intercept and residuals (Table 2).

Considering the T1 samples, the correlation between the unbound concentration of teriflunomide in plasma and the total plasma and RBC teriflunomide concentrations was high ($r = 0.92$ and 0.67, respectively) (Fig. 2). However, the correlation of unbound teriflunomide concentrations with the teriflunomide concentration in CD3$^+$ T cells and CD3$^-$ MNC was noticeably lower (i.e., $r < 0.5$) (Fig. 2).

The median (range) partition coefficient of unbound teriflunomide to CD3$^+$ T cells, CD3$^-$ MNC, and RBC at T1 was 0.243 (0.092–0.632), 0.310 (0.035–0.907), and 23.079 (9.639–37.248), respectively (Fig. 3). At T1, the coefficient representing the partition of unbound plasma teriflunomide to CD3$^+$ T cells was significantly lower than unity ($P < 0.001$).

The partition coefficient for unbound teriflunomide concentrations into CD3$^+$ T cells, CD3$^-$ MNC, or RBC was described by the linear mixed-effect model represented below (Winter, 2013):

$$\text{Partition coefficient} \sim 1 + (1|\text{ID}) + \varepsilon \quad (2)$$

The BSV of the coefficient representing the partition of unbound plasma teriflunomide to CD3$^+$ T cells, CD3$^-$ MNC, or RBC had a trend toward being less than the WSV (Table 2).

TABLE 2

Linear mixed-effect modeling results

The S.D. estimate of the intercept and residuals, respectively, represents the BSV and WSV for the log-transformed teriflunomide concentration metrics and partition coefficient. The 90% confidence intervals (90% CI) of the S.D. estimates have also been represented.

	Intercept (BSV) S.D. Estimate (90% CI)	Residuals (WSV) S.D. Estimate (90% CI)
Teriflunomide Concentration		
CD3$^+$ T cells	0.04 (<0.001–0.40)	0.50 (0.36–0.65)
CD3$^-$ MNC	<0.001 (<0.001–0.50)	0.56 (0.38–0.73)
RBC	0.45 (0.30–0.66)	0.25 (0.18–0.40)
Unbound plasma	0.54 (0.40–0.75)	0.14 (0.10–0.23)
Total plasma	0.53 (0.40–0.75)	0.16 (0.11–0.26)
Teriflunomide partition coefficient		
Unbound to CD3$^+$ T cells	<0.001 (<0.001–0.10)	0.13 (0.11–0.17)
Unbound to CD3$^-$ MNC	0.07 (<0.001–0.19)	0.19 (0.13–0.26)
Unbound to RBC	3.72 (<0.001–7.11)	6.00 (4.25–8.81)

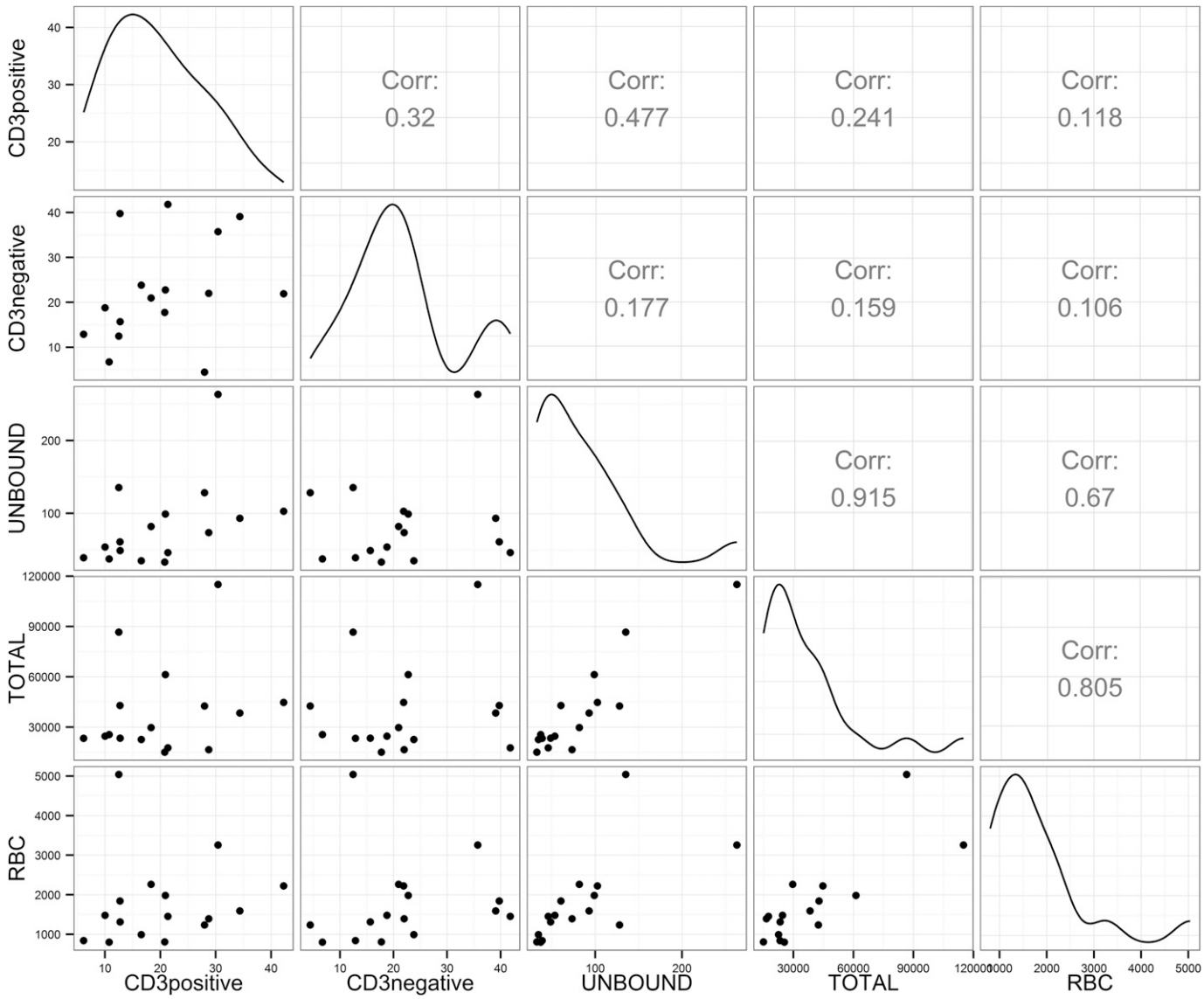

Fig. 2. Correlation matrix of $CD3^+$ T cell, $CD3^-$ MNC, RBC, unbound, and total teriflunomide concentrations at T1. (CD3 positive represents $CD3^+$ T cells, and CD3 negative represents $CD3^-$ MNCs).

Within the eight individuals who provided two matched dose samples of $CD3^+$ T cells, $CD3^-$ MNC, RBC, unbound, and total plasma, the median change (range) in teriflunomide concentration was −29% (−69% –138%), −15% (−73%–133%), −6% (−45%–27%), 14% (−10% –40%), and 1% (−22%–57%), respectively (Fig. 4). Furthermore, the median change (range) in the partition coefficient for unbound plasma teriflunomide into $CD3^+$ T cells, $CD3^-$ MNC, and RBC was −42% (−77%–166%), −19% (−76%–71%), and −21% (−51%–15%), respectively (Fig. 5).

Discussion

In RA patients treated with leflunomide, the concentration of teriflunomide within $CD3^+$ T cells, $CD3^-$ MNC, and RBC was determined for the first time. The partition of teriflunomide into $CD3^+$ T cells and $CD3^-$ MNC was limited, whereas accumulation occurred within RBC. The correlation between unbound plasma teriflunomide concentration and intra-$CD3^+$ T cell and $CD3^-$ MNC concentrations was poor.

The site of action of most small-molecule medicines is within cells, and, although plasma concentrations have been associated with response to therapy, they are merely surrogates for drug concentration at the target site, which, in the case of leflunomide, is mitochondrial DHODH exposure to teriflunomide within $CD3^+$ T cells (Dollery, 2013). Adequate surrogate markers are preferred over measuring drug directly at the target site, as, despite recent advances in mass-spectrometric techniques, there are substantial time, expense, and technical difficulty in measuring intracellular drug concentrations. However, total teriflunomide plasma concentrations, although demonstrating a concentration–effect relationship, are not used clinically as a TCI as concentration thresholds have varied significantly between studies (Chan et al., 2005; Van Roon et al., 2005). This is most likely a reflection of the higher WSV of teriflunomide concentrations within $CD3^+$ T cells that was demonstrated in the present study, and the poor correlation with free plasma teriflunomide concentrations. The most useful markers for TCI generally require low intraindividual variability, so the higher WSV of teriflunomide concentrations in $CD3^+$ T cells in this study does not support its use as a biomarker.

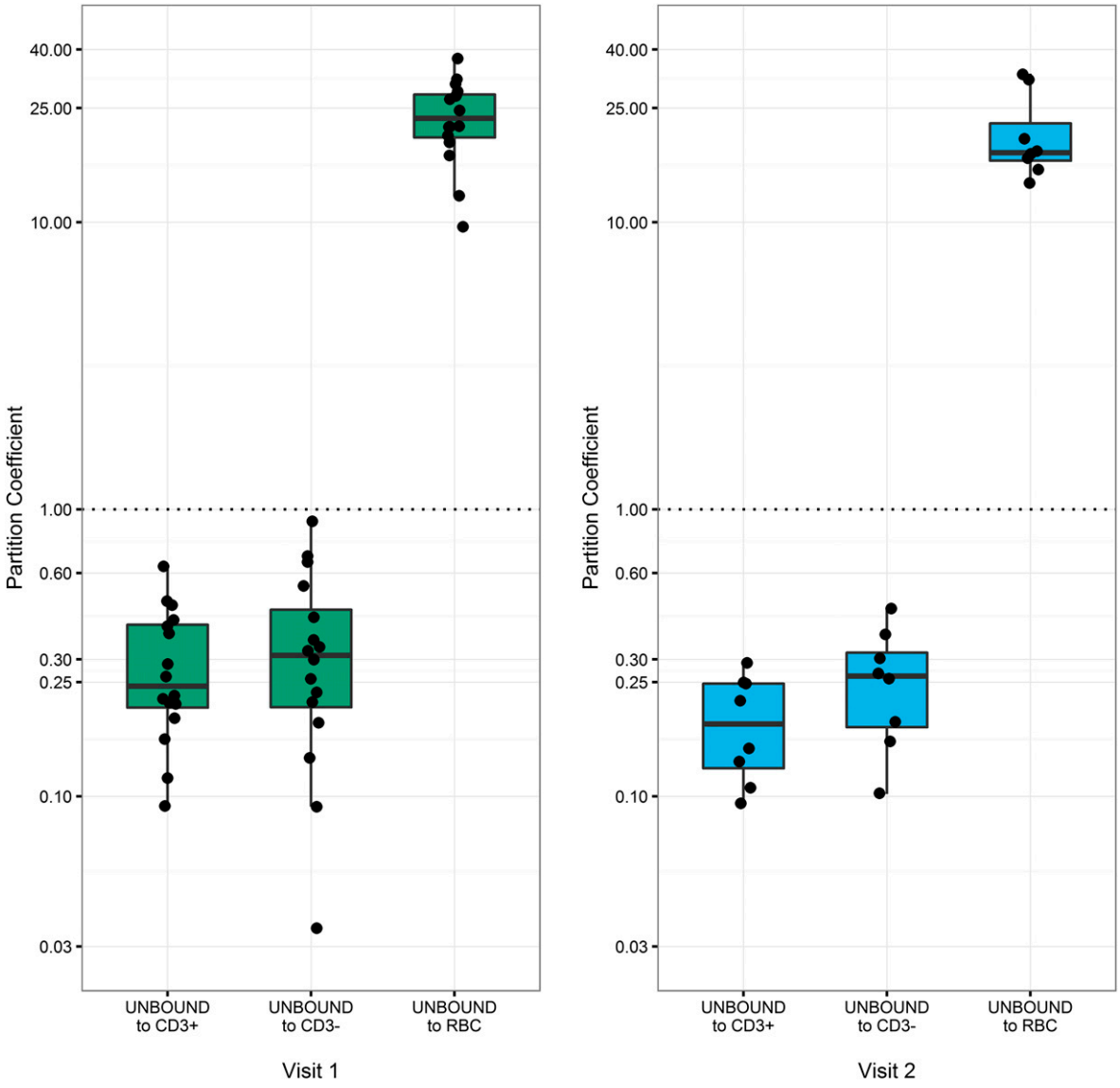

Fig. 3. Box plot of the partition coefficients of unbound teriflunomide to $CD3^+$ T cells, $CD3^-$ MNC, and RBC at T1.

To date the most substantial work on intracellular drug concentrations has been carried out in HIV sufferers. The concentrations of zidovudine, lamivudine, tenofovir, carbovir, and their active metabolites have been explored in MNC, with the intent of identifying the reasons for suboptimal viral suppression (Hawkins et al., 2005; Bazzoli et al., 2011; Wang et al., 2011). In these studies, the intracellular pharmacokinetics have been shown to be more variable than plasma pharmacokinetics, the cause of which has not been fully elucidated (Wang et al., 2011). RBC concentrations of methotrexate polyglutamates have also shown some potential as a suitable efficacy marker in RA (Mohamed et al., 2015). Similar to teriflunomide, methotrexate acts within lymphocytes; however, intralymphocyte concentrations have not been explored due to limitations with analytical sensitivity (Mohamed et al., 2015). Over the past decade, analytical methods have improved (Dollery, 2013), enabling measurement of intra-$CD3^+$ T cell teriflunomide and the subsequent finding of a poor correlation between teriflunomide concentrations in $CD3^+$ T cells and RBC. This highlights the potential dissimilarity between surrogate cell types and target cells, although this may vary from drug to drug according to pharmacochemical properties.

Only unbound unionized drug can passively diffuse into and out of cells. Given teriflunomide is a weak acid (pKa ~5.48) (Wiese et al., 2013), and plasma pH is tightly controlled between 7.38 and 7.42 (Atherton, 2003), approximately 99% (range, 98.8%–98.9%) of unbound teriflunomide will be ionized within the plasma (Po and Senozan, 2001). Despite this, plasma pH may account for some of the WSV of intracellular concentrations, as the permeability of teriflunomide in Caco-2-TC7 cells was decreased by approximately 50% with an increase in pH from 6.5 to 7.4 (http://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/202992Orig1s000ClinpharmR.pdf), whereas predictions indicate that a realistic plasma pH change from 7.38 to 7.42 would halve unbound unionized teriflunomide concentrations from 0.2% to 0.1%. Intracellular pH (pHi) is also important, and it can vary between cell types and even within cell structures (Casey et al., 2010; Chen et al., 2013). Typically, RBC pHi (7.06–7.27) is lower than extracellular pH, and peripheral blood lymphocytes maintain a pHi of approximately 7.17 (Roos and Boron, 1981; Deutsch et al., 1982). Typically, ion trapping occurs when differences between intracellular and extracellular pH result in more ionized intracellular drug. As the pHi of cells assessed in

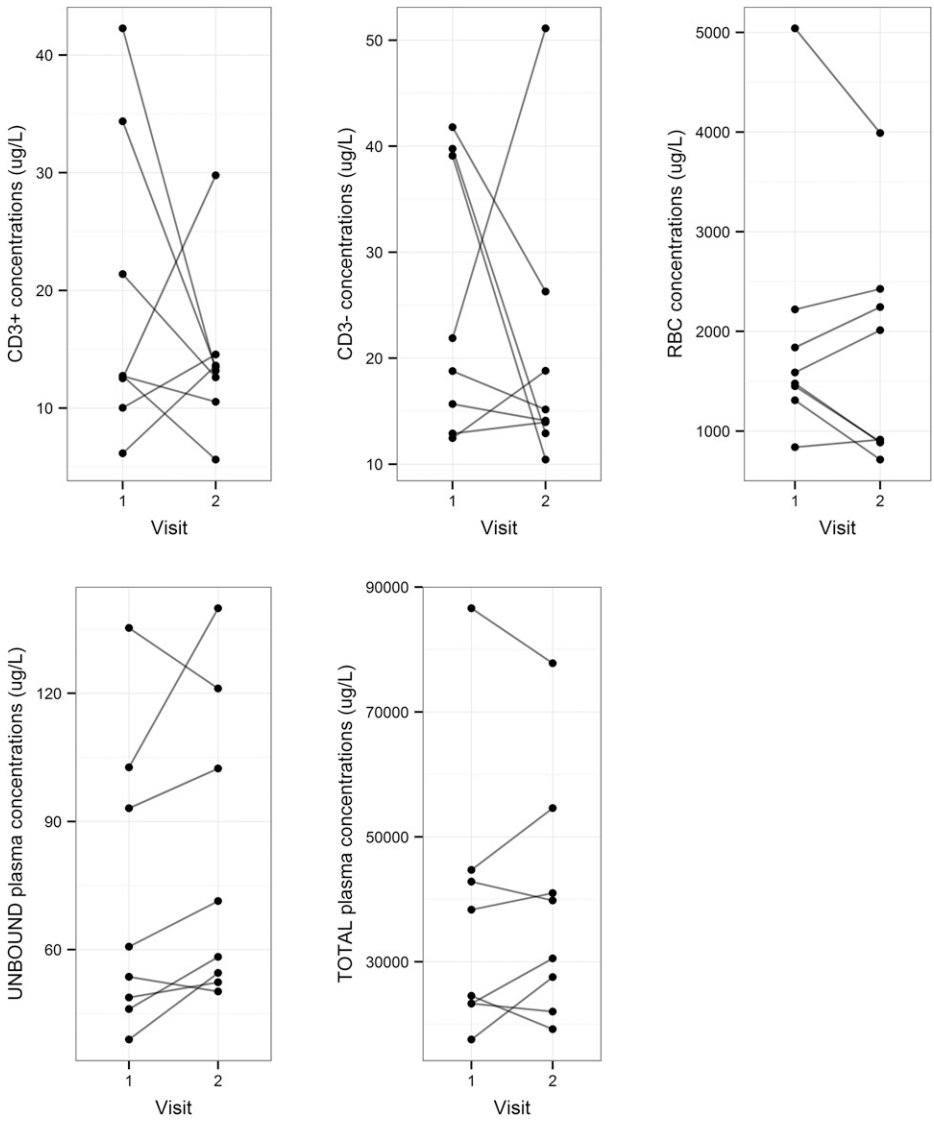

Fig. 4. Matched dose steady-state $CD3^+$ T cell, $CD3^-$ MNC, RBC, unbound, and total teriflunomide concentrations.

this work was expected to be slightly lower than the plasma pH, a minor increase in the unionized percentage of teriflunomide would be expected within cells. Thus, ion trapping, although not directly measured, would appear unlikely to describe the observed intracellular/unbound plasma partition coefficients.

The duration that cells are exposed to unbound unionized teriflunomide is likely to affect whether equilibrium between intracellular and plasma concentrations is reached. RBC have a life span between 70 and 140 days (Franco, 2012), whereas $CD3^+$ lymphocytes have complex maturation profiles and life cycles, depending upon cell type and underlying disease status (Reinherz and Schlossman, 1980; Hellerstein et al., 1999; Appay et al., 2008; Ladell et al., 2008). RBC turnover is relatively constant within an individual; therefore, RBC teriflunomide concentrations will reach a relatively consistent pseudo-steady state. In contrast, circulating T lymphocyte numbers can vary between, and even during a day (Miyawaki et al., 1984). T lymphocytes are also central to the pathogenesis of RA, which has characteristic fluctuations in disease activity, and the resultant effects on cell circulation are currently poorly defined (De Boer et al., 2003; Iikuni et al., 2007; Straub and Cutolo, 2007). Typically, RA increases the turnover rate of lymphocytes, before ultimately resulting in a deterioration of the immune system and T lymphocyte repertoire exhaustion, yet naive or memory $CD4^+$ T lymphocyte numbers are maintained (Koetz et al., 2000; De Boer et al., 2003). It follows that exposure time of $CD3^+$ T cells to unbound unionized teriflunomide may fluctuate between and within individuals. Thus, if the time to reach equilibrium between unbound unionized plasma and intracellular teriflunomide concentrations is longer than the turnover rate of cells, the reported partition coefficient may be influenced by the turnover rate. Consequently, the time to reach equilibrium between plasma and intracellular teriflunomide concentrations should be investigated, particularly in cell subtypes that perish rapidly and at varying plasma pHs.

Different teriflunomide concentrations within $CD3^+$ T cells, $CD3^-$ MNC, and RBC may reflect differences in cellular physiologies between individuals and within different cell types (including lymphocyte subpopulations). For example, there is differing expression of influx and efflux transporters (Chaudhary et al., 1992; Albermann et al., 2005; Prasad et al., 2013), such as the ATP-binding cassette subfamily G member 2, which has been associated with altered teriflunomide plasma concentrations (Kim et al., 2011; Wiese et al., 2012). Varying expression of influx and efflux transporters may also affect the time to reach steady-state intracellular teriflunomide concentrations. Furthermore, mature erythrocytes are rich in hemoglobin, yet barren of organelles (e.g., mitochondria and nucleus), which can express and synthesize

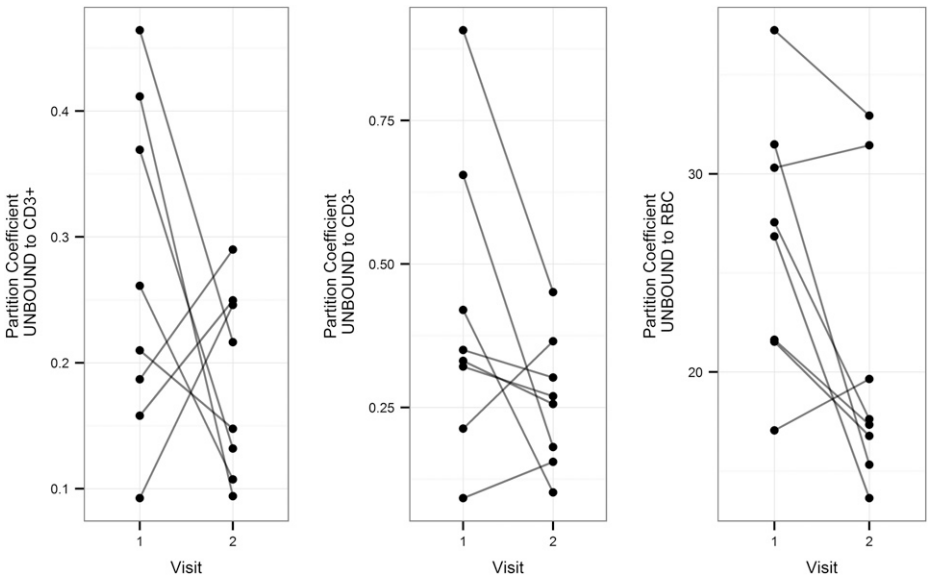

Fig. 5. Matched dose partition coefficients for unbound teriflunomide concentrations to $CD3^+$ T cells, $CD3^-$ MNC, and RBC.

transporters (Wohlrab, 2009). Similar to the high plasma protein binding of teriflunomide, RBC may accumulate teriflunomide through intracellular protein binding, whereas efflux transportation is likely to be lower than within lymphocytes. Conversely, there is potential for differing transporter expression between circulating $CD4^+$, $CD8^+$, $CD16^+$, and NK T lymphocytes (among others) (Boldt et al., 2014), which adds another degree of variability to the concentration of teriflunomide within $CD3^+$ T cells. Therefore, teriflunomide concentrations within T lymphocyte subpopulations should be investigated, as this may reduce the variability and provide a superior efficacy biomarker.

In this study, concentrations were explored within individuals on stable doses of leflunomide, and we assumed that, because teriflunomide has a plasma half-life of approximately 2 weeks (mean ~15.7 days) (Rozman, 2002), steady-state concentrations in cell subtypes would also be reached with 8 weeks of stable dosing. Furthermore, the relationship between teriflunomide concentrations in any matrices with either efficacy or toxicity was not investigated. The isolation of $CD3^+$ T cells is tedious and time consuming, with variations in temperature, washing procedure, and time likely to affect extraction and leaching. Although a strict protocol was used to minimize these affects, the process most likely adds variability to measured concentrations (Wang et al., 2011).

In this study, the sample size was sufficient to determine that $CD3^+$ T cell teriflunomide concentrations are lower than unbound plasma teriflunomide concentrations, which has not been reported previously. Linear mixed-effect modeling was used to assess the between- and within-individual variance of $CD3^+$ T cell, $CD3^-$ MNC, RBC, unbound, and total teriflunomide concentrations. These analyses indicated that the BSV of unbound and total teriflunomide concentrations was greater than the WSV, which aligns with previous literature (Schmidt et al., 2003; Van Roon et al., 2005; Bohanec Grabar, 2009; Rakhila et al., 2011). However, this was in contrast with the results for the $CD3^+$ T cell, $CD3^-$ MNC teriflunomide concentrations, in which there was a trend for the BSV to be less than the WSV. These results will need to be explored further in a larger population analyses; however, at this stage, these preliminary results do not support the use of $CD3^+$ T cell teriflunomide concentrations as a useful marker for TCI. In this study, it was also assumed that the $CD3^+$ T cell, $CD3^-$ MNC, RBC, unbound, and total teriflunomide concentrations, which are physiologic variables, would be log-normally distributed for a population, and that the partition coefficient of free teriflunomide to $CD3^+$ T cell, $CD3^-$ MNC, and RBC teriflunomide would be normally distributed for a population.

For this new field of investigation, it would be desirable to conduct a prospective analysis with a rich data set to analyses both intraday and daily intracellular teriflunomide concentrations, in which the impact of food, concomitant drugs, other environmental factors and transporters is as yet largely unknown. A large prospective analysis that includes patients on stable doses of leflunomide should also be conducted, as this may capture the time to reach intracellular steady state as well as a larger range of $CD3^+$ T cell teriflunomide concentrations. Such a prospective study may also assist the investigation of the relationship between $CD3^+$ T cell teriflunomide concentration and toxicity or efficacy. It would be of particular interest to identify whether $CD3^+$ T cell teriflunomide concentrations are more closely associated with efficacy compared with total concentrations, which to date have demonstrated highly variable thresholds (Chan et al., 2005; Van Roon et al., 2005; Bohanec Grabar, 2009). Because teriflunomide's precise location of action is mitochondrial DHODH (Breedveld and Dayer, 2000), determining teriflunomide concentrations within T lymphocyte mitochondria may also be important for future research, although organelle isolation and analytical sensitivity make such a determination technically difficult at this time.

In conclusion, teriflunomide concentrations within $CD3^+$ T cells displayed higher intraindividual variability and were lower than unbound plasma teriflunomide concentrations. Furthermore, the correlation of $CD3^+$ T cells and RBC teriflunomide concentrations was low, highlighting that, although abundant, RBC appear to be a poor surrogate for drug at the target site. $CD3^+$ T cell subpopulation analysis may be important, as correlation with intracellular $CD3^-$ MNC teriflunomide concentrations was low, highlighting the potential impact of different cellular physiologies, pHi, and circulation profiles.

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Authorship Contributions

Participated in research design: Hopkins, Proudman, Wiese.

Conducted experiments: Hopkins, Moghaddami, Wiese.

Contributed new reagents or analytic tools: Hopkins, Moghaddami, Foster, Proudman, Upton, Wiese.

Performed data analysis: Hopkins, Foster, Upton, Wiese.

Wrote or contributed to the writing of the manuscript: Hopkins, Moghaddami, Foster, Proudman, Upton, Wiese.

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