Cancer Chemother Pharmacol (2010) 66:159–169
DOI 10.1007/s00280-009-1146-y

ORIGINAL ARTICLE

The effects of drug transporter inhibitors on the pharmacokinetics and tissue distribution of methotrexate in normal and tumor-bearing mice: a microdialysis study

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Received: 24 June 2009 / Accepted: 15 September 2009 / Published online: 9 October 2009

Abstract
Purpose To examine methotrexate (MTX) tumor delivery in a mouse model using an in vivo microdialysis technique and to characterize the impact of prior administration of the known transporter inhibitors probenecid and cyclosporine (CsA), alone and in combination, on plasma and tumor pharmacokinetics of MTX.
Methods Different groups of mice were used to evaluate the plasma pharmacokinetics of MTX and the impact of prior administration of probenecid and/or CsA on the plasma pharmacokinetics. Xenografted nude mice were used for microdialysis experiments to measure the subcutaneous (SC), peri- and intratumoral pharmacokinetics of MTX without and with coadministration of probenecid, CsA, and both probenecid and CsA.
Results The SC dialysates in pre-treated groups demonstrated a delayed disappearance and an enhanced MTX exposure. Similar effects were observed in the tumor peripheral zone. However, this increase was less pronounced. The central tumor findings demonstrated that CsA had a more significant impact on the enhancement of MTX exposure. Probenecid did not increase the exposure of MTX inside the tumor, but caused a longer half-life of central MTX.
Conclusions This study revealed significant differences in the relative estimated PK parameters of the plasma, SC, peri-, and intratumoral zones. Additionally, this study demonstrated that the coadministration of MTX with CsA can enhance the intratumoral exposure levels of the drug, whereas coadministration of MTX with probenecid alone, or with a combination of probenecid and CsA, increases intratumoral half-life.

Keywords Microdialysis · Methotrexate · Pharmacokinetics · Cancer chemotherapy

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Introduction

The therapeutic efficacy of anticancer drugs is critically dependent upon the maintenance of adequate dose intensity in a target tissue. Despite many chemotherapeutic successes in recent years against hematological cancers, anticancer drugs have had a more limited impact on solid tumors. Although intracellular mechanisms of tumor cell resistance to anticancer drugs are widely recognized as a key component in cancer therapeutic failure, more attention is focused on drug transport and delivery to tumors as the sources of treatment failures [1]. Clinical drug resistance still remains a major obstacle to the successful treatment of cancer, and one of the mechanisms by which cancer cells become resistant to chemotherapy is the

expression of several members of the ATP-binding cassette (ABC) superfamily of membrane transporters. There are primarily three ABC transporters associated with the multidrug resistance phenomenon; P-glycoprotein (Pgp), multi-drug resistance proteins (MRPs/ABCC), and breast cancer resistance protein (BCRP/ABCG2). These transporters have broad and overlapping substrate specificities, and they efflux major drugs currently used in cancer chemotherapy [2]. Several recent reviews have discussed the pharmacological, physiological, and crucial roles of these transporters in limiting the bioavailability and tumor penetration of their substrate drugs [3–8].

Methotrexate (MTX) has had a crucial role in cancer chemotherapy, in combination with other chemotherapeutic agents, in the treatment of various human malignancies such as breast cancer, osteosarcoma, acute lymphocytic leukemia, choriocarcinoma, lung cancer, and chronic myeloid leukemia [9]. The pharmacological activity of MTX is attributed to its reversible inhibition of dihydrofolate reductase (DHFR), a key intracellular enzyme in folic acid metabolism which leads to disruption of the synthesis of essential nucleic acids for the replication of DNA and RNA [10].

Previous attempts to utilize plasma levels of cytotoxic drugs as indicators of tumor responses to therapy have generally failed, suggesting that plasma concentration–time profiles for anticancer drugs are not necessarily a good measure of concentration–time profiles at target sites, i.e. the vicinity of tumor cells [11]. Additionally, preliminary clinical investigations in breast cancer patients have demonstrated that there is no association between the plasma concentration of a chemotherapeutic agent and the tumor response, but that concentrations of chemotherapeutic agents in a tumor may correlate with the response to chemotherapy [12, 13]. Studies have also shown that anticancer drugs do not distribute uniformly in the body, but rather reach varying concentrations in different tissues. These tissue concentrations are more predictive of clinical outcomes than plasma concentrations in some cases [14–16].

The therapeutic responses of tumors to MTX can be hindered by suboptimal tissue/target site concentrations and development of drug resistance due to active drug efflux. This is mediated by several members of the ABC superfamily of membrane transporters, including MRPs/ABCC and BCRP/ABCG2 [17]. The first direct evidence that MRPs are likely to have a significant role in MTX disposition was provided by Masuda et al. in 1997, when they demonstrated that MTX is transported into the bile in wild type rats, but not in mutant Eisai hyperbilirubinemic rats (EHBR) which have a hereditary defect in the function of MRP2 transporters [18]. Three years later in 2000, Volk et al. [19] showed that cancer cells with marked BCRP overexpression were cross-resistant to MTX. Both MRPs and BCRP are located primarily in the plasma membrane, where they actively extrude a variety of structurally diverse drugs and metabolites, including MTX [5].

In recent decades, there has been a growing interest in the use of the microdialysis technique to evaluate the disposition of anticancer agents in tumors and tissues of interest, in order to identify factors associated with a lack of tumor response [1]. More specifically, it is often the interstitial tissue space that is most closely related to the site of action of a drug. Microdialysis samples the unbound fraction of the drug in the interstitial space, which is in equilibrium with biophase membrane barriers and receptors [20].

Over the past decade, quantitative microdialysis has been used to examine the brain penetration of drugs in numerous studies and a great number of publications have examined the CNS microdialysis studies to characterize the distribution of a variety of drugs to the brain as well as the roles of transporters as they relate to the brain distribution of the drugs [21–26]. Additionally, in animal models, Bihorel et al. [27] demonstrated the proof of principle that inhibiting multi-drug efflux transporters can enhance drug delivery to the brain for selected compounds and can thereby improve tumor response.

Quantification of drug delivery to tumor tissues using the microdialysis technique can be an attractive way to assess whether sufficient amounts of anticancer drugs can reach their targets. The number of studies conducted in animal tumor models to investigate target site disposition is very limited, and to our knowledge, no study has yet been conducted to investigate the effects of coadministration of anticancer agents with selective transport inhibitors on the intratumoral exposure level.

The objective of this study was, therefore, to evaluate the impact of prior administration of the known MRP and BCRP inhibitors probenecid and cyclosporine A (CsA), alone and in combination, on the pharmacokinetic profiles and exposure levels of MTX subcutaneously, and at both peri- and intratumoral zones of an MDA-MB-231 breast tumor in athymic nude mice.

Materials and methods

Drugs and chemicals

Methotrexate [(±) amethopterin], probenecid, cyclosporin, NaH_2PO_4, and Na_2HPO_4 were obtained from Sigma–Aldrich (St. Louis, MO, USA). Purified water was obtained from a Nanopure ultrapure water system (Barnstead, Thermo Fisher Scientific, Boston, MA, USA). All other reagents were of analytical grade.

Microdialysis system

Linear microdialysis probes (CMA30, 10 mm cuprophane membranes, 6,000 Da cutoff) and peripheral perfusion fluid T1 were purchased from CMA Microdialysis (Chelmsford, MA, USA). The peripheral perfusion solution was delivered, using a 1 mL glass microsyringe at ambient temperature, by a Harvard apparatus pump, and perfusates were collected manually at specified time intervals.

Animals

Animals were obtained from Charles River Laboratories, Wilmington, MA. All animals underwent a period of environmental acclimation and were fed ad libitum. A 12 h light: 12 h dark cycle was maintained. Animals were euthanized immediately after study procedures by CO_2 asphyxiation. All animal-related experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Charles River Laboratories, Shrewsbury, MA.

Analytical HPLC method

Samples were analyzed for MTX concentrations by high-performance liquid chromatography (HPLC). The published HPLC method for MTX analysis [28] was modified to improve both resolution and sensitivity. The HPLC system consisted of an ESA autosampler, dual-piston pump, Phenomenex Luna C_{18} (2) column (150 × 4.6 mm, 5 μm particles), a mobile phase of 0.1 M sodium dihydrogen phosphate: methanol (75:25 v/v) at pH 7.0, 1 mL/min flow rate, and UV detection at 370 nm. The modified HPLC method was validated for precision and reproducibility. Limits of detection and limits of quantification in different matrices were determined. Plasma proteins in blood samples were precipitated with methanol (1:1 methanol to sample ratio) and the dialysate samples were injected into the HPLC system without further modification.

Cell culture

Tumor cells from a breast adenocarcinoma cell line (MDA-MB-231) were purchased from ATCC® (Manassas, VA, USA) and were grown in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin solution, in 25 cm^2 culture flasks maintained at 37°C. Cells were harvested during their logarithmic growth phase by trypsinization, and appropriate aliquots of cell suspension were added to new culture vessels with 6–8 mL of complete growth medium. The medium was renewed two to three times per week.

Tumor model

Subcutaneous, peri-, and intratumoral dialysate concentrations of MTX were obtained from nude mice bearing an (MDA-MB-231) xenograft. Injections of 10^7 cells were made into the right lateral thorax of athymic nude mice. Throughout the study, the length (L) and width (W) of each developed tumor was measured in millimeters with calibrated vernier calipers, where L was the longer of the two dimensions. The corresponding tumor weight was calculated using the experimental formula: weight of tumor (mg) = $(L \times W^2)/2$. Animals were selected for use in the study when tumors reached a palpable size of approximately 600 mg. This was generally within 4–5 weeks of inoculation. In total, 12 tumor-bearing mice were randomly selected and assigned to three different treatment groups and a control group for the experiment.

Microdialysis probe calibration

In vitro calibration

Microdialysis probe recovery rates were evaluated in vitro and in vivo. The objective of the in vitro calibration study was to determine the diffusion characteristics of MTX at different concentrations by the CMA30 dialysis probe. For this, each linear probe (2 μL/min flow rate, $n = 3$) was exposed to various concentrations of MTX (1, 10, and 100 μM) at 37°C with gentle agitation. Samples from the probe outlet were collected every 10 min for 2 h and the resulting concentrations of MTX were determined. Subsequently, the relative recoveries were calculated by determining the ratio of the probe outlet concentration of MTX to the concentration of the external solution of MTX in the proximity of the probe, and expressing the ratio as a percentage $(C_{out}/C_{medium}) \times 100$.

In vivo calibration

Relative recovery (RR) of MTX in vivo was evaluated using the retrodialysis method. In retrodialysis, or reverse dialysis, the drug itself is added to the perfusate fluid and its in vivo loss is used as a measure of in vivo recovery. The principle of this method relies on the assumption that the diffusion process is quantitatively equal in both directions across the semi-permeable membrane. Therefore, the relative loss of the drug into the tissue, before the experiment, is representative of its recovery, or relative gain, from the tissue during the experiment. Thus, based on this principle, known concentrations of MTX solution were included in the perfusate fluid and the rate of drug disappearance through the membrane was subsequently

calculated as the in vivo recovery. C_{out} (MTX concentration in the dialysate) was measured and RR (%) was calculated as the in vivo recovery according to the following equation:

$$RR(\%) = 100 - [100 \cdot (C_{dialysate}/C_{perfusate})]$$

The in vivo recovery assessment based on the retrodialysis method was performed using three different concentrations of MTX (1, 10, and 100 μM) in nude mice (three animals/concentration). The mean in vivo recovery value was used to correct the concentrations of MTX in dialysate samples.

Calculations for microdialysis experiments

The concentrations of MTX in the dialysate samples obtained in microdialysis experiments were corrected based on the mean in vivo recovery obtained from the in vivo recovery study. Estimates of the interstitial concentrations were calculated based on the in vivo relative recovery by the following equation:

$$\text{Interstitial concentration} = 100 \cdot \text{dialysate concentration}/\text{in vivo recovery value.}$$

Pharmacokinetic experiments

Plasma pharmacokinetic study

A group of male CD-1 mice ($n = 20$, 2 animals/time point, Group 1) was used to evaluate the plasma pharmacokinetics of MTX following a single intravenous dose of MTX in PBS (200 mg/kg, 20 mg/mL concentration). Samples were collected via cardiac puncture after animals were euthanized with CO_2 at 1, 3, 5, 15, 30, 60 min, 2, 4, and 6 h post dose. A refrigerated centrifuge (3,500 rpm for 10 min) was used to process whole blood to plasma. Plasma samples were transferred directly to appropriate tubes and stored at -70 ± 10°C until HPLC analysis. Similarly, another group of male CD-1 mice ($n = 26$, 2 animals/time point, Group 2) was used to evaluate the impact of probenecid (50 mg/kg) and/or CsA (10 mg/kg) when used as a pretreatment on the plasma pharmacokinetics of MTX following intravenous administration (200 mg/kg). All pretreatment solutions were given 30 min prior to dosing with MTX.

Dialysate pharmacokinetics study

Nude mice with human (MDA-MB-231) breast tumor cell xenografts ($n = 3$ per group) were used for microdialysis experiments to measure the subcutaneous, peri-, and intratumoral pharmacokinetics of MTX (200 mg/kg), administered without (Control Group) and with probenecid (50 mg/kg, Group 3), CsA (10 mg/kg, Group 4), or both probenecid and CsA (Group 5). To each animal was administered medetomidine (0.75 mg/kg, i.p.) and ketamine (70 mg/kg, i.p.), prior to subcutaneous probe insertion. Microdialysis probes were placed subcutaneously (SC) and into the peripheral and central areas of each tumor. Serial extracellular fluid (ECF) samples were collected every 10 min for 3 h after dosing. Animals received MTX (200 mg/kg) via an i.v. injection and all pretreatments were given 30 min prior to MTX dosing.

Pharmacokinetic data analysis

The plasma concentration–time data of MTX were subjected to a two-compartmental pharmacokinetic analysis using WinNonlin® professional edition software, version 5.2 based on the non-linear regression method of Gauss–Newton with the Levenberg/Hartley modification. The applied weighing scheme in the plasma data modeling was weighted by 1/observed Y^2.

The mean concentration–time points were fitted to the equation; $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$ where α and β ($\alpha > \beta$) are hybrid first-order rate constants for the distribution and elimination phase, respectively, and A and B are intercepts on the y-axis for each exponential segment of the curve.

The dialysate concentration–time data was subjected to a non-compartmental pharmacokinetic analysis based on the linear trapezoidal (linear interpolation) method. Major pharmacokinetic parameters were obtained and interstitial AUC of free peri- and intratumoral MTX was defined as the main outcome variable. The ratios for SC and tumor penetration were determined as AUC_{sc}/AUC_{plasma} and AUC_{tumor}/AUC_{plasma}, respectively. Additionally, one-way analysis of variance (SigmaStat, Version 3.5) with the Holm-Sidak post-hoc test was used to compare differences among the various zones (SC zone was considered as a reference to compare other tumor zones) and to compare differences between the three pretreatments in each zone. Student t test was also used to compare the differences in PK parameters.

A p value of less than or equal to 0.05 was considered to be statistically significant.

Results

In vitro calibration

In vitro recovery experiments demonstrated that a stable equilibrium was attained after 30 min, and that the relative recovery of MTX after equilibration was constant at three different concentrations: 1, 10, and 100 μM (Fig. 1). The mean in vitro recovery for MTX was 39 ± 3.5%.

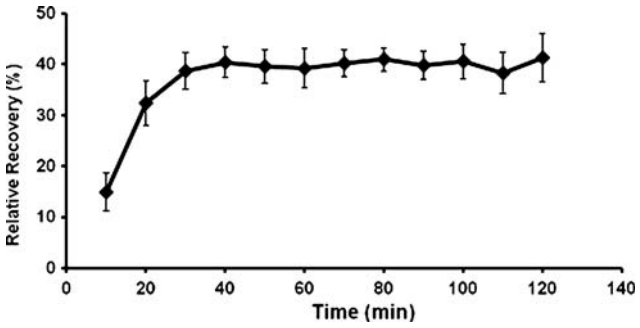

Fig. 1 In vitro relative recovery (%) of MTX. Time-dependent equilibration of 100 μM MTX during in vitro microdialysis. Microdialysis was performed at 37°C with gentle agitation. Data are expressed as mean ± SE ($n = 3$)

In vivo calibration

In vivo data indicated that equilibrium conditions in nude mice were reached after 40 min and that recovery was concentration-independent. The mean in vivo recovery for all concentrations of MTX was 25 ± 4%.

Plasma pharmacokinetics

An overlay plot combining the plasma concentration (mean ± SE) versus time for MTX in male CD-1 mice following nominal intravenous doses of 200 mg/kg, coadministered without (Group 1) and with inhibitors (Group 2) is shown in Fig. 2. Summary statistics of the pharmacokinetic parameters of MTX are compared in Table 1. The plasma concentrations of MTX showed a bi-exponential PK profile, with a rapid decline over the initial 15 min followed by a slower second phase. The initial distribution phase was clearly distinguishable due to the frequency of initial sampling times. The pharmacokinetic parameter estimates in Group 1 (mean ± SE) were $t_{1/2} = 2.4 \pm 1$ min, $C_{max} = 1{,}691 \pm 922$ μg/mL, $K_{10} = 0.3 \pm 0.1$ 1/min, and AUC = 5,827 ± 989 min μg/mL. The systemic clearance of MTX was observed to be 34 ± 6 mL/min per kg.

In Group 2, pretreated with inhibitors, the plasma concentrations of MTX also showed a bi-exponential profile with a rapid decline over the initial 15 min, followed by a slower second phase; however, the disappearance of MTX was delayed. The MTX pharmacokinetic parameter estimates (mean ± SE) were $t_{1/2} = 8 \pm 0.1$ min, $C_{max} = 1{,}247 \pm 19$ μg/mL, $K_{10} = 0.08 \pm 0.001$ 1/min and AUC = 14,608 ± 97 min μg/mL. The systemic clearance of MTX (14 ± 0.09 mL/min per kg) was observed to be reduced by one-half and the level of MTX exposure was higher than in the control group.

Dialysate pharmacokinetics

A plot combining the dialysate concentration (mean ± SE) versus time curve for MTX in the SC space, peripheral, and central zones of the tumor in the control group, following a nominal, single intravenous dose of MTX (200 mg/kg) is depicted in Fig. 3. Dialysate data are corrected for in vivo recovery. The individual profiles reflect the unbound concentration of MTX in the subcutaneous extracellular space, peripheral, and central zones of the tumor, respectively. The concentration of MTX in the subcutaneous space peaked with a delay of 30 min and had a prolonged terminal half-life ($\lambda_{1/2} = 26 \pm 2$ min). The other PK parameter estimates were $C_{max} = 65 \pm 4$ μg/mL, $T_{max} = 30$ min and $AUC_{inf} = 2{,}945 \pm 399$ min μg/mL. The ratio of MTX exposure in the subcutaneous space to that of plasma (AUC_{sc}/AUC_{plasma}) was 0.4, indicating that only 40% of the systemic plasma exposure level was achieved subcutaneously. C_{max} and T_{max} at the peripheral zone of the tumor were comparable to those of the SC space; however, the exposure level ($AUC_{inf} = 2{,}077 \pm 156$ min μg/mL) was decreased and the terminal half-life ($\lambda_{1/2} = 31 \pm 7$ min) was more prolonged than in SC.

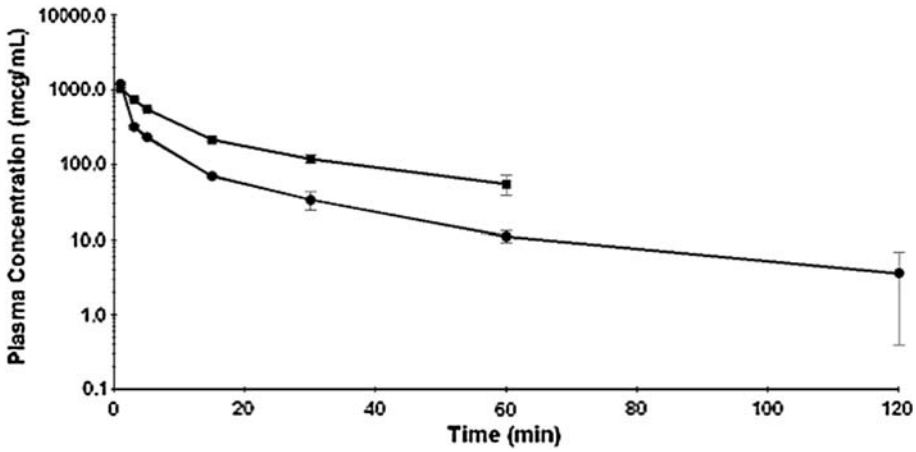

Fig. 2 Plasma concentration versus time curve in mice following a nominal intravenous dose of 200 mg/kg MTX without (*filled circle*) and with inhibitor pre-treatments conditions (*filled square*). Data are expressed as mean ± SE

Table 1 Comparison of estimated PK parameters of MTX following nominal intravenous doses of 200 mg/kg in mice without and with pre-treatment conditions

Plasma PK parameters	MTX	MTX + Inhibitors
AUC (min μg/mL)	5,827 ± 989 (16)	14,608 ± 97* (1)
CL (mL/min per kg)	34 ± 6 (17)	14 ± 0.09* (1)
$\alpha_{1/2}$	1.3 ± 0.5 (37)	3 ± 0.1 (3)
$\beta_{1/2}$	25 ± 5 (19)	27 ± 0.6 (2.3)
$t_{1/2}$ (min)	2.4 ± 1 (43)	8 ± 0.1* (2)
K_{10} (1/min)	0.3 ± 0. 1 (43)	0.08 ± 0.001 (2)
C_{max} (μg/mL)	1,691 ± 922 (54)	1,247 ± 19 (1.5)
V_{ss} (mL/kg)	628 ± 209 (33)	384 ± 6 (1.5)

Values are Mean ± SE; CV% shown in parentheses

* $p \leq 0.05$ based on student t test between groups

The C_{max} and AUC_{inf} in the center of the tumor were significantly decreased ($C_{max} = 14 \pm 3$ μg/mL, $AUC_{inf} = 1{,}323 \pm 193$ min μg/mL) and the terminal half-life ($\lambda_{1/2} = 63 \pm 8$ min) was 2.4-fold longer than that of SC. The ratio of systemic MTX exposure in the center of the tumor to that of plasma ($AUC_{center}/AUC_{plasma}$) was found to be 0.2, indicating that only 20% of the systemic plasma exposure and one-half of the SC exposure level was achieved in the center of the tumor.

The SC disappearance of MTX in groups receiving inhibitor pretreatments was delayed, as compared to the control animals (Figs. 4, 5, 6). The terminal elimination half-lives (mean ± SE) in Groups 3–5 were 40 ± 1, 41 ± 16, and 68 ± 24 min, respectively, compared to that of the control animals (26 ± 2 min), which indicated a longer SC half-life for MTX in all treatment groups. This increase was more pronounced (2.6-fold) in Group 5 (probenecid + CsA) than in Groups 3 and 4. Also, a significant difference in MTX exposure level (AUC_{inf}) was observed between pretreated groups and control animals. The AUC_{inf} in Groups 3–5 were 3,671 ± 1,064, 4,220 ± 1,168, and 9,661 ± 1,686 min μg/mL, respectively, and compared to that of the control animals (2,945 ± 399 min μg/mL), indicated a higher exposure level of SC MTX in all treatment groups. The increase was also more pronounced (3.2-fold) in Group 5 (probenecid + CsA) than in Groups 3 and 4. A similar pattern was also observed in SC C_{max}; when Groups 3–5 (67 ± 27, 75 ± 8, and 86 ± 7, μg/mL, respectively) were compared to the control animals (65 ± 4 μg/mL), a higher C_{max} was indicated in Groups 4 and 5. The increase was most pronounced in Group 5, in which animals received pretreatment with both probenecid and CsA, while in Group 3, which received the pretreatment of probenecid alone, C_{max} was comparable to that of the control group.

The peripheral disappearance of MTX was also delayed in all treatment groups when compared with the control animals. The peripheral terminal elimination half-lives in Groups 3–5 were approximately twice as long as that in the control animals, indicating a longer half-life of MTX at the tumor periphery in all treatment groups. Contrary to the SC site, this half-life increase at the peripheral site was smaller in Group 5 (probenecid + CsA) than in the other treatment groups. Also, a significant difference in the MTX exposure level (AUC_{inf}) was observed between Groups 4 and 5, and the control animals. The AUC_{inf} in Groups 3–5 were 2,100 ± 341, 2,847 ± 1,039, and 5,052 ± 1,750 min μg/mL, respectively, compared to 2,076 ± 156 min μg/mL in the control animals. This indicates that a higher tumor peripheral MTX level was attained in Groups 4 and 5, while the exposure level in Group 3 was similar to that in the control animals. This increase was also more pronounced (2.5-fold) in Group 5 (probenecid + CsA). C_{max} and T_{max} at this site were comparable to those of the controls and no significant increase was observed.

The central tumor disappearance of MTX was delayed in Groups 3 and 5, but was similar in Group 4

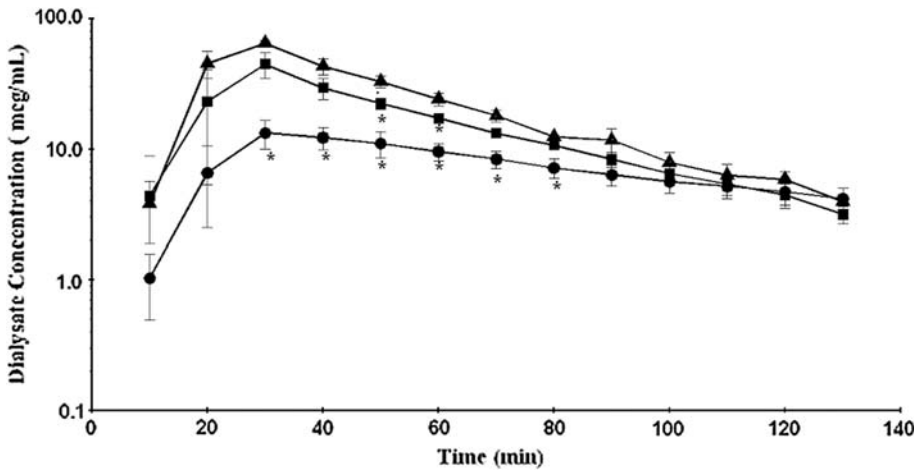

Fig. 3 Subcutaneous (*filled triangle*), peripheral (*filled square*), and central (*filled circle*) tumor dialysate concentrations versus time following a nominal intravenous dose of 200 mg/kg MTX in tumor-bearing mice. Individual data points represent the mean ± SE. Data were analyzed by one-way ANOVA and Holm-Sidak post-hoc method. *Significantly different from the value of SC zone at each time point, $*p < 0.05$

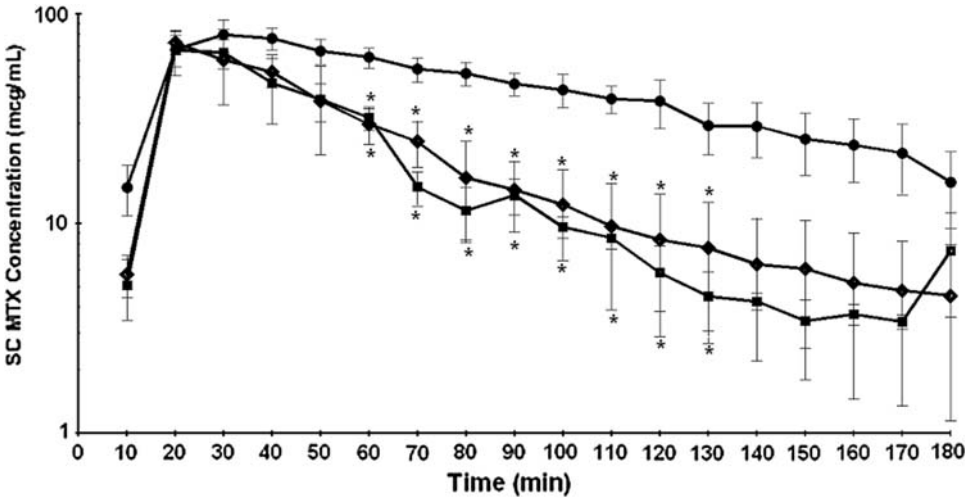

Fig. 4 Effect of transporter inhibitors on SC dialysate concentrations of MTX versus time in three different pre-treatment groups (*filled circle* Probenecid+ CsA, *filled square* Probenecid, and *filled diamond* CsA) following an IV dose of 200 mg/kg MTX administered in PBS (data are corrected for recovery). Individual data points represent the mean ± SE. *Significantly different from the value of (*filled circle* Probenecid+ CsA) pretreatment at each time point, $*p < 0.05$

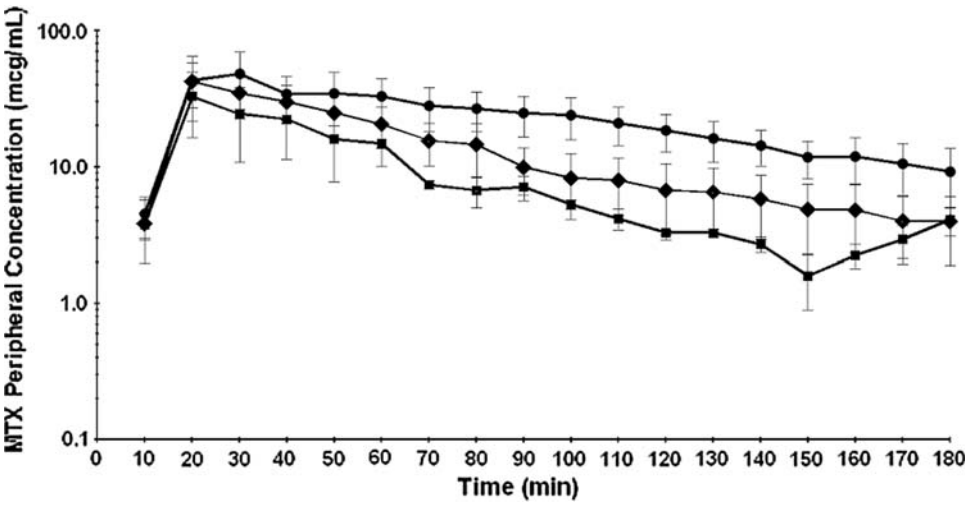

Fig. 5 Effect of transporter inhibitors on peripheral dialysate concentrations of MTX versus time in three different pre-treatment groups (*filled circle* Probenecid+ CsA, *filled square* Probenecid, *filled diamond* CsA) following an IV dose of 200 mg/kg MTX administered in PBS

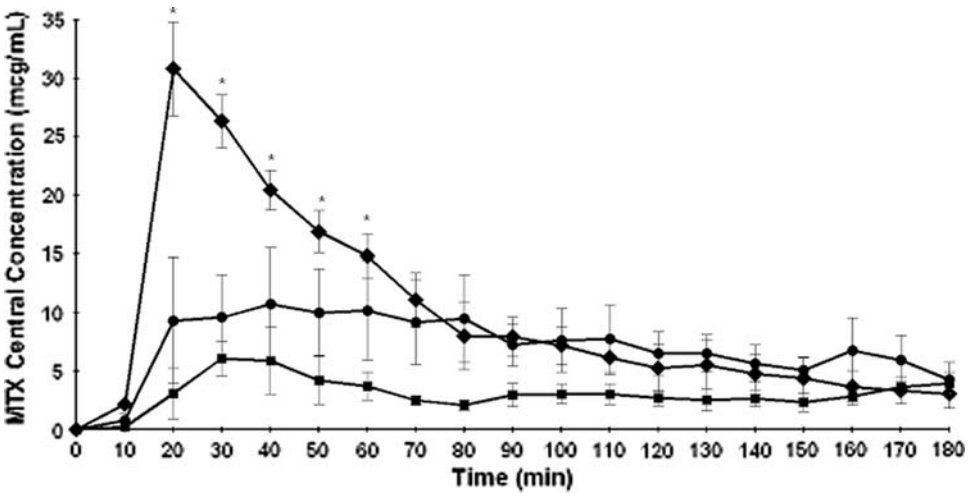

Fig. 6 Effect of transporter inhibitors on central dialysate concentrations of MTX versus time in three different pre-treatment groups (*filled circle* Probenecid+ CsA, *filled square* Probenecid, *filled diamond* CsA) following an IV dose of 200 mg/kg MTX administered in PBS. Individual data points represent the mean ± SE. *Significantly different from the value of other pre-treatment groups, $*p < 0.05$

(CsA pretreatment only) to that of the control animals, indicating a longer half-life of MTX in the center of the tumor only in treatment Groups 3 and 5. However, significant increases in the MTX exposure level (AUC_{inf}) and C_{max} were observed in Group 4 (twofold increase in AUC and C_{max} compared to those of the controls). The AUC_{inf} in Group 5 indicated a lesser increase over the control group (1,703 ± 404 min μg/mL vs. 1,323 ± 192 min μg/mL),

while the Group 3 AUC_{inf} was comparable to the control group. T_{max} was also significantly decreased in Group 4 compared to the other groups. The results for T_{max} in Groups 3–5 were 80 ± 27, 19 ± 3, and 40 ± 6 min, respectively, compared to 33 ± 3 min in the control animals.

Discussion

A key question in delivery of an anticancer drug to a tumor is whether the anticancer drug reaches the intended site of action and achieves sufficient concentrations to result in pharmacological response. In this study, we characterized and compared the impact of prior administration of the known MRP and BCRP inhibitors, probenecid and CsA, alone and in combination, on the pharmacokinetic profiles and exposure levels of MTX in an experimental breast tumor model by subcutaneous and intratumoral microdialysis sampling following i.v. administration of MTX. As a commonly used drug for treating autoimmune or inflammatory diseases, CsA has been demonstrated to be a broad-spectrum modulator for multidrug resistance proteins such as P-gp, BCRP, MRP1, and MRP2 [5, 29]. Anionic drugs such as probenecid have been shown to interact with MRP2 and MRP1 [30, 31]. Although more selective BCRP, P-gp, and MRP1/2 inhibitors exist, CsA and probenecid are widely used standard inhibitors of these multi-drug transporters in clinical practice to reverse multi-drug resistance (MDR) in therapy and to improve drug disposition [29, 32, 33]. Furthermore, using broad spectrum inhibitors for some clinical applications might be advantageous because more than one transporter; BCRP, MRP1/2, and P-gp can be blocked at the same time to improve drug disposition and penetration into the tumor.

In vitro recovery experiments were performed prior to in vivo recovery experiments, to investigate whether diffusivity of MTX is, indeed, independent of concentration. In vitro recovery experiments indicated that equilibrium conditions were reached within 30 min and were independent of absolute MTX concentration. These results were in line with previous findings by Muller et al. [14] and Ekstrom et al. [28]. The mean in vitro recovery for MTX was 39.3%, similar to that reported by Muller et al. [14], but higher than the in vitro recoveries reported by Ekstrom et al. and Dukic et al. [28, 34]. This could be due to the longer length of the membrane and the specific conditions employed in our experiment. These findings needed to be verified in vivo to evaluate whether in vitro recovery is relevant to in vivo conditions. When the probe is implanted into a tissue which has a more complicated microenvironmental matrix, the in vivo recovery is altered relative to the in vitro recovery, as the diffusion coefficient and mass transport of a compound can be very different in living tissue. This can be affected by other factors such as tissue tortuosity, blood flow, microviscosity, limited volume fraction in the extracellular space of the extracellular fluid, interaction of analyte with structural macromolecules of the tissue, and the degree of tissue vascularization and metabolism [1, 20]. Our in vivo recovery data indicated that equilibrium conditions in nude mice were reached after 40 min and that recovery was concentration-independent and did not closely approximate the in vitro recovery. It is also noteworthy that the measured in vitro and in vivo recoveries are unique to the specific experimental setup employed.

The estimated plasma PK parameters in our experiments were lower than those reported by Lobo et al. [35] in Swiss Webster male mice (clearance: 2.4 ± 0.4 L/kg per hour; half-life: 33.8 ± 6.5 min) after an i.p. bolus of 3–600 mg/kg MTX. Our estimated clearance value was similar to that reported by Osman et al. [36] in female Swiss albino mice after i.p. bolus of 50 mg/kg MTX. The disappearance of MTX from plasma when coadministered with inhibitors was slower than in control animals, as indicated by the reduced systemic clearance of MTX and a higher level of exposure. These results corroborate previous clinical findings on the increased efficacy of low to moderate doses of MTX when coadministered with CsA in rheumatoid arthritis patients. This is due to elevated plasma levels (+18%) and decreased plasma clearance of MTX, resulting from the inhibitory effect of CsA on renal blood flow, which alters the pharmacokinetics of MTX [32, 37, 38]. The prolonged and enhanced MTX plasma levels can also be attributed to the inhibitory effect of probenecid on biliary secretion and renal tubular transport of MTX, as previously reported in both rats and monkeys [39, 40].

The feasibility of using microdialysis for the in vivo measurement of MTX was first investigated in 1994 by Ekstrom et al. [28] in a rat model, in a study which demonstrated that microdialysis can be used to provide reproducible pharmacological data in various tissues. Three years later, the same authors studied the differences in MTX distribution within the same xenograft tumor of subcutaneous human osteosarcoma, grown in nude rats given MTX infusion (37.5 mg/kg/3 h) with the aid of microdialysis probes inserted into the center and periphery of the tumor. Significant differences were observed in microdialysis data for MTX levels within sub-compartments of the tumor, as the central part of the tumor was exposed to a lower MTX concentration during the infusion [41]. Our findings of differences in the relative estimated PK parameters of the plasma, SC space, peri- and intratumoral sites are in agreement with their findings. Additionally, we observed that despite the decrease in the exposure level of MTX toward the center of the tumor,

the elimination half-life became longer, indicating a slower efflux and elimination of the drug from the center of the tumor. These differences in the relative estimated PK parameters of the plasma, SC space, peri- and intratumoral sites might be attributed to the potential heterogeneity of the tumor tissue. Comparisons of histological sections of tumors, using staining techniques, have revealed differences between the central and peripheral portions of the tumors. For instance, necrosis in the central part of a tumor has been found to be more evident than in the periphery [41]. Moreover, the structure of vasculature and the degree of vascularization in different parts of the tumor could be variable, resulting in significant differences in the estimated PK parameters at different sites. These recognized differences in vascular permeability of different parts of the tumor, together with the frequent absence of an anatomically well-defined and functioning lymphatic network, can affect drug uptake and retention inside the tumor, resulting in a prolonged drug terminal half-life within the tumor subcompartments. The elevated level of intratumoral pressure and reduced drug efflux generally demonstrated in the literature might also result in a diminished convection of the drug and a reduced degree of drug exposure [11].

To our knowledge, the effects of transporter inhibitors on intratumoral differences of MTX exposure have not been studied previously. Since microdialysis measures unbound fraction of the drug in the interstitial space, there are several factors which can determine the "measured" concentration of MTX in interstitial fluid (ISF) near the tumor. These include the systemic exposure of unbound drug in plasma (plasma protein binding), blood flow to the tumor, the degree of drug binding in tumor ISF, and the degree of equilibrium between intracellular fluid and ISF along with intracellular protein binding. One would anticipate that the inhibition of the efflux pumps on the plasma membrane of cancer cells could increase intracellular retention of MTX, leading to the alteration of the equilibrium of both MTX cellular influx and efflux along with their relationship with the accumulated form of intracellular MTX (polyglutamylated MTX) [17]. Therefore, the degree of inhibition of the efflux pump is expected to influence MTX concentration in ISF. Although it is beyond the scope of the present study, information about dose-dependent inhibition of MTX efflux following administration of inhibitors would allow quantification of the effects pump inhibitors on MTX concentrations in ISF.

The SC dialysate results from groups pretreated with transporter inhibitors demonstrated a delayed disappearance, a longer half-life, and an enhanced MTX exposure level in all pretreated groups. A more pronounced increase was observed in the group pretreated with both probenecid and CsA than in the groups pretreated with either probenecid or CsA alone. This can be attributed to the inhibitory effect of CsA on renal blood flow [37] and the effects of probenecid to decrease the biliary secretion and renal tubular transport of MTX [39, 40].

In peripheral tumor dialysates from groups pretreated with transporter inhibitors, similar effects were observed; there were delayed peripheral disappearances and longer MTX half-lives in all treated groups. In contrast to the SC space, these effects in the periphery were less pronounced in the group pretreated with the combination of probenecid and CsA than in groups pretreated with either probenecid or CsA alone. Also, a comparable exposure level of MTX was observed between the group pretreated with probenecid and the control group.

Our findings from central tumor dialysates in groups pretreated with transporter inhibitors demonstrated that CsA had a more significant impact on the enhancement of MTX exposure level (twofold increase in AUC_{inf} and C_{max} compared to controls) than the combination of probenecid and CsA (28% increase in AUC_{inf}) and probenecid alone (comparable to the controls); however, the terminal elimination half-life in the CsA-treated group was comparable to that of the control group. Probenecid, on the other hand, did not increase the exposure level of MTX inside the tumor, but contributed to a longer half-life of central MTX than that resulting from exposure to combined probenecid and CsA or to CsA alone.

These observations can be attributed to both systemic and local (tumor) inhibitory effects of CsA and probenecid and the relative distribution of each transporter involved. The inhibitory effect of CsA on BCRP is dependent upon BCRP expression. Human (MDA-MB-231) breast tumor cells are known to have a high expression of BCRP transporters. Moreover, mice have displayed the highest expression of BCRP in kidney [5]. CsA not only has an inhibitory effect on renal tubular excretion and clearance reduction (secondary to renal capillary constriction) but also is expected to have a local inhibitory effect. Furthermore, CsA can block oxidation of MTX (aldehyde oxidase enzyme) to its relatively inactive metabolite,7-OH-MTX, all of which potentiate higher systemic and local exposure of MTX. However, the local inhibitory effect of probenecid on breast tumor cells might be quite limited with probenecid exerting its systemic inhibitory effects primarily by decreasing biliary secretion and renal tubular transport of MTX [39, 40]. Probenecid, on the other hand, when used in combination with CsA might serve as a competitive inhibitor of CsA systemic action leading to a smaller central tumor exposure than seen with CsA alone.

Other possible explanations for these observations might be differences in the expression and distribution of particular transporters in the center and periphery of the tumor, compared to those in the SC space. Other

contributing factors might be the relative impact of each particular transporter, its cross-interaction with CsA and probenecid, and the considerably broad specificity of CsA inhibiting BCRP on one hand and Pg-p, MRP1, and MRP2 on the other hand [5]. The challenge still remains to clarify the relative impact of each particular transporter, cross-interactions, potential interactions with other drug processing systems, such as uptake carriers of drugs, and metabolizing enzymes. Further studies are needed to better characterize the impact of inhibitors on MTX efficacy and toxicity. In particular, additional experimental evidence to determine the relative contribution of each efflux transporter using more selective inhibitors such as elacridar and tariquidar may be required.

A logical extension of the present work should include investigation of the relationship between the measured local (tumor) concentrations and a pharmacodynamic parameter such as a reduction in tumor size of 50% or more with direct measurement of tumor diameters in order to establish a local PK/PD relationship. This information could help to predict effective anti-cancer drug concentrations in human tumors and could be useful in elucidating the relationship between local drug exposure and the therapeutic response in the treatment of superficially located solid tumors.

Conclusion

In these studies, utilization of the in vivo microdialysis technique to examine MTX delivery in a human breast cancer model revealed significant differences in the relative estimated PK parameters of the plasma, subcutaneous space, peri- and intratumoral target sites, all of which indicated that in vivo microdialysis is a valuable tool to study drug biodistribution in small animals. Additionally, it was revealed that coadministration of MTX with CsA can enhance the intratumoral exposure levels of the drug, whereas coadministration of MTX with probenecid alone, or probencid plus CsA, causes a longer intratumoral half-life. Also, the effects of drug transporter inhibitors on plasma/subcutaneous exposure levels are not a predictor of the effects of these inhibitors on the intratumoral exposure levels.

These types of studies provide detailed information about drug exposure levels achieved in tumor tissues, and could ultimately help to select novel cytotoxic compounds with favorable biodistribution and tumor penetration characteristics, and also to explain why some patients are drug resistant. This information will help to optimize dosing and administration schedules of solid tumor therapies by themselves, or in combination with drug transporter inhibitors.

Conflict of interest statement None.

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