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ORIGINAL ARTICLE

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Pharmacodynamic properties of methotrexate and Aminotrexate™ during weekly therapy

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Abstract 4-amino-pteroyl-glutamic acid (Aminotrexate™; AMT) has several advantages over the related antifolate methotrexate (MTX), including greater potency, complete oral bioavailability, and greater accumulation by leukemic blasts in vitro. We compared the pharmacodynamic properties of AMT (given orally at 4 mg/m^2 in two divided doses per week) and MTX (100 mg/m^2 in four divided doses per week) among children with acute lymphoblastic leukemia. We find AMT and MTX to have equivalent penetration into the bone marrow compartment of these patients, as indicated by the steady-state concentrations within mature red blood cells (RBCs). However, MTX concentrations in the cerebrospinal fluid after oral dosage are significantly greater than AMT. To confirm these clinical observations, mice were treated four weekly injectons of AMT or MTX, at a 1:20 dosage ratio, and tissue antifolate content was then determined over the subsequent 22 days. We confirm the selective exclusion of AMT from the CNS compartment, while showing equivalent accumulation of AMT and MTX in the RBCs, liver, spleen, kidneys and testes. Finally, we demonstrate that AMT, MTX, and their predominant polyglutamate species are equipotent inhibitors of their target intracellular enzyme dihydrofolate reductase, emphasizing the critical nature of steady-state tissue accumulation in determining the relative cytotoxic potency of these two antifolates.

Keywords Aminopterin · Methotrexate · Polyglutamates · Neurotoxicity · Acute lymphoblastic leukemia · Rheumatoid arthritis

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Introduction

Folic acid antagonists (antifolates) are cytotoxic drugs used as antineoplastic, antimicrobial, anti-inflammatory, and immune-suppressive agents. Although several antifolates have been developed, methotrexate (4-amino-4-deoxy-10-*N*-methyl-pteroylglutamic acid; MTX) is the antifolate with the most extensive history and widest spectrum of clinical use. MTX is an essential drug in chemotherapy regimens used for patients with acute lymphoblastic leukemia (ALL), osteosarcoma, choriocarcinoma, lymphoma, breast cancer, bladder cancer, and head and neck cancer. In addition, it is used at lower doses for patients with non-malignant diseases such as rheumatoid arthritis, psoriasis, and graft-versus-host disease.

4-Amino-4-deoxy-pteroyl-glutamic acid (aminopterin; now being developed under the trade name Aminotrexate™; AMT) is an antifolate in the same class as MTX. AMT has several advantages over MTX, including 20–40 times greater clinical potency, [9, 13], complete oral bioavailability, [33] and higher V_{max}/K_m for the enzyme folylpolyglutamate synthetase,[15] leading to greater accumulation and metabolism by patients' leukemic blasts [43] in vitro. We are in the process of testing whether AMT can be safely substituted at its MTD (4 mg/m^2 /week in two divided doses orally) [33] for MTX in multi-agent therapy for children newly diagnosed with ALL at a high risk (HR) of relapse. To date, 29 patients have received AMT on this protocol, and clinical toxicity has been no greater than that observed among patients at a standard risk (SR) of

relapse, who receive identical therapy, except that MTX is the only antifolate given.[7].

While this trial proceeds, we have been comparing the pharmacokinetic and pharmacodynamic properties of these two antifolates, focusing on the penetration into relevant tissues. For example, red blood cell (RBC) MTX concentrations are thought to reflect steady-state penetration of MTX into the bone marrow compartment [8, 23], and have been related to the clinical outcome among patients with ALL [16, 36–40] or rheumatoid arthritis [1, 10, 11, 24]. Similarly, antifolate accumulation by hepatocytes[6, 22, 49] may relate to variations in hepatic toxicity. MTX-related neurotoxicity may be caused by the effects of antifolate penetration into the central nervous system [32, 46]. For these reasons, we have studied the accumulation of AMT and MTX into these and other body compartments and organs after repeated exposure in both mice and patients.

In our patients with ALL, we now show equivalent exposure to the bone marrow, as assessed by RBC concentrations, at AMT doses 25-fold lower than those of MTX. However, we demonstrate lower concentrations of AMT in the cerebrospinal fluid (CSF). Consistent with our clinical experience, we find equivalent accumulation of the two antifolates into peripheral organs of the mice at an AMT:MTX dosage ratio of 1:20, but again we find decreased penetration of AMT relative to MTX in the brain parenchyma.

Finally, since the cytotoxicity of classical antifolates like AMT and MTX seems to depend on the inhibition of the intracellular target enzyme dihydrofolate reductase (DHFR), we have compared the ability of AMT and MTX to inhibit DHFR in vitro. We show that AMT, MTX, and their predominant polyglutamate species are similarly potent inhibitors of DHFR, emphasizing the critical nature of steady-state tissue accumulation in determining the relative cytotoxic potency of these two antifolates.

Methods

Clinical trial: CINJALL

The Cancer Institute of New Jersey Acute Lymphoblastic Leukemia trial (CINJALL) is a pilot study testing the toxicity of AMT as part of the multi-agent therapy for children with newly diagnosed ALL at a HR of relapse. The protocol and informed consent were approved by the Institutional Review Board (IRB) of the University of Medicine and Dentistry of New Jersey, and opened in March, 2001. The treatment scheme is shown in Table 1. Children with ALL at SR of relapse by NCI criteria (age 1–10 years, WBC < 50,000/μl, no CNS or testicular disease, and no unfavorable cytogenetics) are treated with multi-agent chemotherapy, including divided-dose oral MTX as previously published, 25 mg/m^2 /dose for four doses six hours apart [48]. Those at HR of relapse (all others) are non-randomly assigned to receive a single Delayed Intensification and oral AMT at its MTD, 2 mg/m^2/dose for two doses 12 hours apart [33], in place of oral MTX. Patients with the T-lineage disease are not excluded, and T-lineage alone is not used as a HR criterion.

Divided-dose oral antifolate therapy is given weekly in Consolidation and every other week in Intensive Continuation. A single antifolate dose is given weekly in Continuation. No MTX or AMT is given intravenously. Rules for dose modification due to toxicity are identical on the two arms. In Consolidation, systemic therapy is withheld if the absolute neutrophil count (ANC) is below 500/μl or platelets are below 75,000/μl.

RBC MTX and AMT

In the 12-week Consolidation phase of CINJALL, oral antifolate is given weekly. Patients at an SR of relapse by NCI criteria are given MTX 25 mg/m^2 /dose for four doses, 6 hours apart, or for only three doses on weeks including intrathecal chemotherapy. Those at a HR of relapse receive AMT 2 mg/m^2 /dose for two doses, 12 h apart. Samples are collected for the analysis of RBC antifolate at the end of the 12-week cycle, because RBC MTX is known to reach a steady-state after a minimum of 4–6 weeks of unchanged oral therapy [18]. Well-mixed whole blood was diluted with four volumes of extraction buffer (50 mM tris base, 10 mM EDTA, 150 mM mercaptoethanol, pH 8) and heated to 100°C for 20 min. After centrifugation, the supernatant was aliquoted and frozen at −80°C until analyzed. RBC MTX and AMT were assayed as previously described, using radioligand binding assays [16, 20, 21].

CSF MTX and AMT

During consolidation, intrathecal chemotherapy is given every other week, 4–12 h after an oral antifolate (the second of two AMT doses, 12 h apart, or the third of three MTX doses, 6 h apart). CSF (1–4 ml) is collected, prior to the administration of intrathecal chemotherapy, for analysis of antifolate concentrations. During weeks when systemic therapy is held for hematologic toxicity, CSF is still collected and intrathecal therapy is given. After centrifugation, the supernatant is aliquoted and frozen at −80°C until analyzed. CSF folate and antifolate are assayed in an undiluted CSF sample, using radioligand binding assays with a lower limit of sensitivity of 1–2 nM [21].

Tissue penetration in mice

All experiments were approved by the Institutional Animal Care and Use Committee and were carried out in accordance with the European Union guidelines for the handling and use of laboratory animals. The tissue

Table 1 Outline of therapy given on the Cancer Institute of New Jersey Acute Lymphoblastic Leukemia (CINJALL) trial for children with newly diagnosed ALL

	Standard risk (Age 1–10 yrs, WBC < 50 k, CNS neg, no unfavorable cytogenetics)		High risk (All others)	
Induction (4 weeks)	Daunomycin	60 mg/m^2 IV day 1	Daunomycin	60 mg/m^2 IV day 1
	Dexamethasone	3 mg/m^2 /dose po twice daily days 1–28	Dexamethasone	3 mg/m^2 /dose po twice daily days 1–28
	Vincristine	1.5 mg/m^2 IV weekly × 4 (max. dose 2 mg)	Vincristine	1.5 mg/m^2 IV weekly × 4 (max. dose 2 mg)
	L-asparaginase	10,000 units/m^2 IM days 2,8,11,15,18,22	L-asparaginase	10,000 units/m^2 IM days 2,8,11,15,18,22
	IT Triples	days 1, 29	IT Triples	days 1, 15, 29
Consolidation (12 weeks)	MTX	25 mg/m^2/dose po q6 h for 4 doses each week.	AMT	2 mg/m^2/dose po q12 h for 2 doses each week.
	Leucovorin	5 mg/m^2 × 2 doses, 48 hours after the first MTX, on non-LP weeks.	Leucovorin	5 mg/m^2 × 1 dose, 48 hours after the first AMT, on non-LP weeks.
	6-Mercaptopurine	37.5 mg/m^2 /dose po twice daily	6-Mercaptopurine	37.5 mg/m^2 /dose po twice daily
	IT Triples	Triple Intrathecal therapy weeks 3, 5, 7, 9, and 11	IT Triples	Triple Intrathecal therapy weeks 3, 5, 7, 9, and 11
Delayed Intensification (8 weeks)	n/a		Vincristine	1.5 mg/m^2 IV day 1 of weeks 1,2,3 (max. dose 2 mg)
			Dexamethasone	3 mg/m^2 /dose po twice daily × 21 days, starting day1, week 1
			Daunomycin	25 mg/m^2 IV on day 1 of weeks 1, 2, 3
			6-Thioguanine	60 mg/m^2 /dose daily × 14 days, weeks 5, 6
			Cyclophosphamide	1,000 mg/m^2 IV on day 1, week 5
			Cytarabine	75 mg/m^2 IV or SQ daily × 4, days 2–5 of weeks 5 and 6
			IT Triples	Day 2, week 5 and 6
Intensive Continuation (8 × 8-week cycles)	MTX	25 mg/m^2 /dose po q6 h for 4 doses, weeks 1,3,5,7	AMT	2 mg/m^2 /dose po q12 h for 2 doses, weeks 1,3,5,7
	Leucovorin	5 mg/m^2 /dose po q12 h × 2 starting 48 h after the start of MTX	Leucovorin	5 mg/m^2 × 1 dose, 48 hours after the first AMT
	6-mercaptopurine	37.5 mg/m^2 /dose po twice daily	6-mercaptopurine	37.5 mg/m^2 /dose po twice daily
	L-asparaginase	10,000 IU/m^2 IM < 6 hours after 2nd AMT dose, weeks 1,3,5,7 of the first four cycles of Intensive Continuation	L-asparaginase	10,000 IU/m^2 IM < 6 hours after the last MTX dose, weeks 1,3,5,7 of the first four cycles of Intensive Continuation
	Vincristine	1.5 mg/m^2 IV day 1 of week 8 (max. dose 2 mg)	Vincristine	1.5 mg/m^2 IV day 1 of week 8 (max. dose 2 mg)
	Dexamethasone	3 mg/m^2 po twice daily × 7 days, starting day 1, week 8	Dexamethasone	3 mg/m^2 po twice daily × 7 days, starting day 1, week 8
	IT Triples	week 8 of each cycle	IT Triples	week 8 of each cycle + week 3 of the first four cycles
Continuation (until 30 mos post remission)	MTX	40 mg/m^2 /dose po once, weeks 1–7	AMT	2 mg/m^2 /dose po once, weeks 1–7
	6-Mercaptopurine	37.5 mg/m^2 /dose po twice daily	6-Mercaptopurine	37.5 mg/m^2 /dose po twice daily
	IT Triples	week 8 of each cycle	IT Triples	week 8 of each cycle

IT Triples refer to the combination of MTX, cytarabine and hydrocortisone, dosed by age and given intrathecally

distribution and anti-inflamamtory effects of AMT and MTX were tested in a murine air-pouch model of inflammation [31]. Male CDI Swiss mice, 10–15 week old, were given four weekly intraperitoneal injections of vehicle (0.9% saline; control), AMT (0.05 mg/kg) or MTX (1 mg/kg). This ratio of AMT to MTX (1:20) was chosen based on preliminary data showing these doses to be equally effective in reducing inflammation in a murine model of arthritis. After the fourth injection, a cohort of the animals from each treatment arm (AMT, MTX, and vehicle control) was sacrificed on post-exposure days 1, 3, 5, 8, 12, and 22. Organs and fluids were removed from each animal and frozen. Specifically, antifolate concentrations were examined within the blood, liver, spleen, kidneys, testes, and brain. A portion of each tissue was weighed and homogenized in an extraction buffer (10 mM Tris, 10 mM EDTA, 150 mM mercaptoethanol, pH 8, at 4°C). Antifolate content was then quantitated using a radioligand binding assay, as previously described [21]. Mean tissue values in the control mice were subtracted from those of the treated mice. Although this background "noise" was generally less than 10% of that found in the tissues of treated animals, it accounted for 25–30% of the total apparent AMT in the brain, because the total accumulation was so low that a large amount of the extract needed to be assayed. Results are reported as pmol per gram wet tissue weight ± the standard error of the mean (SEM).

Inhibition of human DHFR

Recombinant human DHFR was obtained as a generous gift from Dr. Joseph R. Bertino [41]. DHFR enzyme activity was assayed as previously described [12], by measuring the decrease in absorbance at 340 nm caused by the conversion of NADPH and dihydrofolate to $NADP^+$ and tetrahydrofolate [30]. Assays were performed in a constant volume (1 ml), at room temperature with the MATS assay buffer system, pH 7.4, with excess dihydrofolate (final concentration, 150 μM) and NADPH (final concentration, 150 μM). Varying concentrations of AMT, MTX, or their respective polyglutamate species were allowed to equilibrate with enzyme for 10 minutes prior to the addition of the substrate (dihydrofolate). For each inhibitor, every concentration tested was repeated at least three times. For each inhibitor, the IC_{50} (concentration required to inhibit enzyme activity by 50% compared to the control) was calculated using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA).

Results

RBC antifolate among patients with ALL

At the end of 12 weeks of oral AMT therapy (4 mg/m^2 / week), the mean (±SEM) RBC AMT (277 ± 38 pmol/ml RBC; $n = 16$) was not statistically different from the mean RBC MTX (271 ± 50 pmol/ml RBC; $n = 13$; $P > 0.5$) after 12 weeks of oral MTX (100 mg/m^2 /week). This RBC MTX is similar to that seen in a historical cohort of 57 patients (287 ± 21) on the same oral MTX regimen, analyzed by the same laboratory [16].

CSF antifolate concentrations after oral therapy

Two to six hours after oral therapy, CSF MTX concentrations (mean ± SE = 26.3 ± 5.0 nM; $n = 45$; Fig. 1) were significantly greater than CSF AMT (3.56 ± 0.83 nM; $n = 64$; $P < 0.0001$, two-tailed t-test). During weeks when systemic antifolate therapy was held due to hematologic toxicity, mean CSF antifolate concentrations were found to have fallen near the lower limits of detection (< 2 nM) of the assay in both MTX (3.3 ± 1.4 nM; $n = 10$) and AMT (2.9 ± 1.5 nM; $n = 18$) arms.

Tissue penetration of AMT and MTX in Mice

Blood was collected after four weekly injections of either AMT (0.05 mg/kg) or MTX (1 mg/kg). On post-exposure day 1, the mean ± SEM whole blood AMT (58.1 ± 8.7 pmol/ml whole blood; $n = 6$; Fig. 2a) was not significantly different from the whole blood MTX (69.0 ± 4.9 pmol/ml blood; $n = 6$: $P = 0.3$). By post-exposure day 22, both RBC AMT and MTX had fallen by 62% (to 21.6 and 26.2 pmol/ml blood, respectively), consistent with the 30 day life span of a murine erythrocyte [17].

Similarly, the penetration of AMT and MTX into the liver and spleen, two additional organs with hematopoietic elements in mice, was identical (Fig. 2b, c). Unlike blood, spleen, and the kidney content, liver AMT and MTX did not decline significantly over the post-treatment observation period (6 and 15% reduction by day 22, respectively; $P > 0.05$ for each comparison).

The testes and kidneys were examined as organs at risk for leukemic relapse and antifolate toxicity, respectively. No significant difference between the AMT and MTX penetration into these organs was observed (Fig. 2 d, e).

In contrast to these peripheral organs, the brain parenchyma contained significantly less AMT than MTX at each post-treatment time point examined (Fig. 2f; $P \leq 0.0003$ for each comparison, two-tailed t test).

Dihydrofolate reductase

As has been previously demonstrated [26], we found longer chain MTX-polyglutamates to be somewhat (approximately two fold) more potent DHFR inhibitors than the parent compound (Fig. 3). However, we found

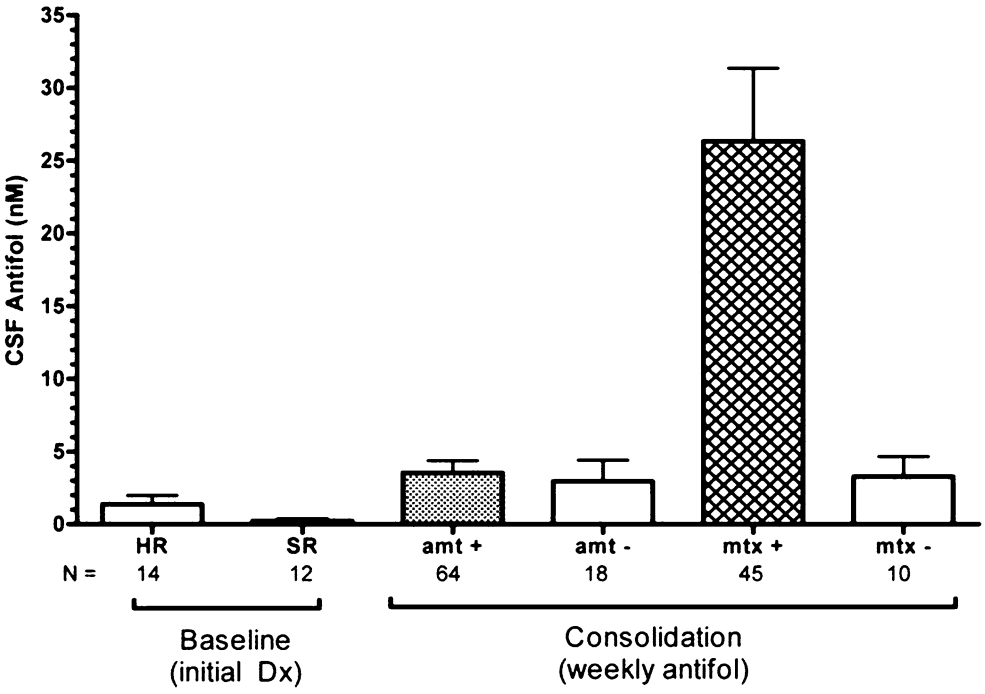


Fig. 1 CSF antifolate concentrations after oral dosage in children with ALL. Median ± SE. CSF antifolate measured at the baseline (initial diagnosis) for patients at high risk (HR) and standard risk (SR) of relapse, and during Consolidation. Abbreviations: "AMT + " : CSF AMT 2–6 h after oral AMT, 2 mg/m^2 . "MTX + " : CSF MTX 2–6 h after oral MTX, 25 mg/m^2 . "AMT-", "MTX-" : median CSF antifolate on weeks when systemic therapy was held for hematologic toxicity

no significant difference between the potency of AMT and MTX, nor between MTX-diglutamate and AMT-diglutamate (the predominant AMT-polyglutamate species in most cell types) [25, 35].

Discussion

The metabolism and pharmacodynamics of MTX have been studied extensively. Consistent with their structural similarity, AMT and MTX have quantitatively similar properties at most intracellular targets. A notable exception is folylpolyglutamate synthase (FPGS), the cytosolic and mitochondrial enzyme that adds a tail of glutamate residues to the reduced folates and classical antifolates, making them highly negatively charged, thereby trapping them in the cell. As a purified enzyme, FPGS exhibits a marked preference for AMT ($V_{max}/K_m = 62$) over MTX ($V_{max}/K_m = 3$) [15], a major factor accounting for the greater cellular accumulation of AMT relative to MTX. Experimental data showed that AMT is taken up four times more rapidly than MTX, and at steady-state attains a similar intracellular concentration to MTX at one-tenth the extracellular concentration in both murine leukemia cells [42] and patient blasts [43] in vitro.

We confirm that AMT and MTX are similarly potent inhibitors of DHFR. We are in the process of testing the relative abilities of AMT and MTX-polyglutamates to inhibit additional intracellular target enzymes, such as thymidylate synthase and the folate-requiring enzymes necessary for de novo purine synthesis, glycinamide ribonucleotide and aminoimidazole carboxamide ribonucleotide transformylases. Regardless of the new results, if DHFR is the primary target of classical antifolates [3], our observation highlights the critical nature of differences in steady-state tissue accumulation in determining the relative cytotoxic potency of AMT and MTX.

Clinically, accumulation of MTX by leukemic blasts has been linked to the outcome by more than one investigator [29, 47]. Steady-state RBC antifolate concentration, a surrogate marker for bone marrow exposure in the preceding 4–8 weeks, correlates with the treatment outcome in patients with leukemia [37] or rheumatoid arthritis [10]. Equivalent human and mouse whole blood values therefore support the pharmacodynamic equivalence of AMT and MTX at a dosage ratio between 1:20 and 1:25.

In the peripheral tissues, accumulation of AMT and MTX is similar even when extracellular concentrations of AMT are 20–25 times lower. Similar results are observed with patients' leukemic blasts in vitro [43]. Antifolate concentrations within the brain parenchyma, however, seem to be proportional to those in the extracellular fluid. Since CSF concentrations of both AMT and MTX are approximately 1% of the plasma levels, an AMT to MTX dosage ratio of 1:20 results in proportionally lower CSF AMT concentrations, and therefore lower brain parenchyma AMT concentrations.

The observation of equivalent peripheral accumulation raises a paradox, in light of previous data that intracellular AMT is primarily found primarily as a diglutamate [25, 35], while MTX is metabolized to longer chain polyglutamates. It is generally accepted that longer chain folyl- or antifolyl-polyglutamates are preferentially retained within the cell longer and are more toxic [2, 19, 34]. Nevertheless, at 20–25-fold lower

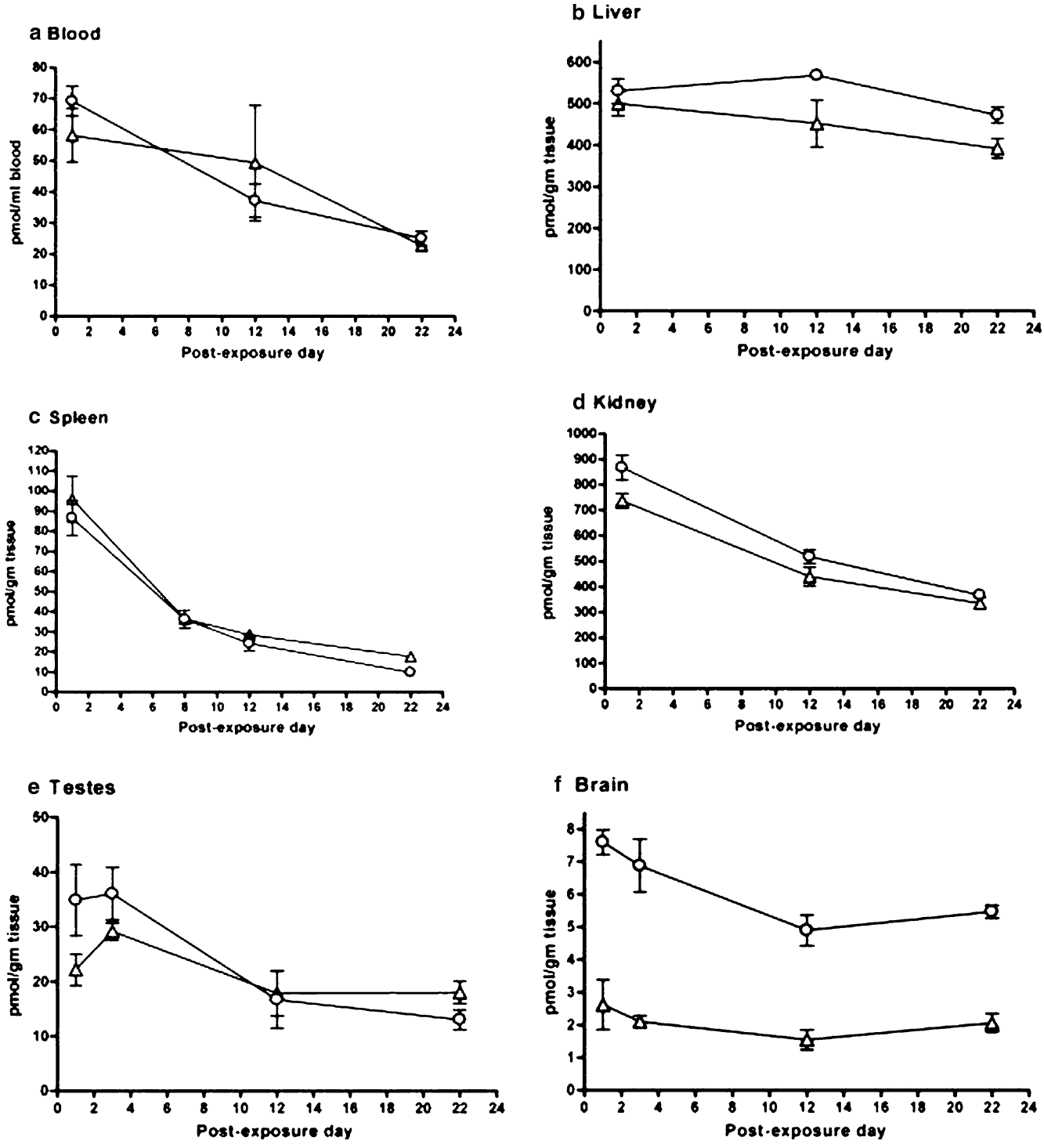
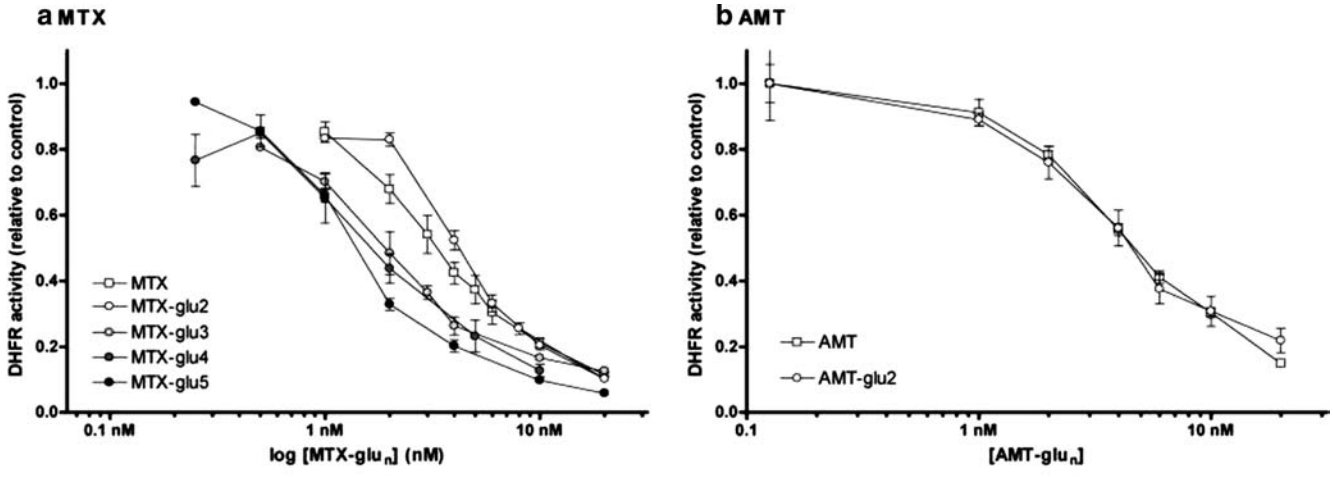


Fig. 2 Tissue content of AMT and MTX, after four weekly doses. After four weekly doses of saline, AMT (0.05 mg/kg) or MTX (1 mg/kg), six mice in each group were sacrificed at each of the post-exposure time points indicated. Blood (**a**), livers (**b**), spleens (**c**), kidneys (**d**), testes (**e**) and brains (**f**) were removed and the antifolate content measured. The mean background levels in saline treated mice were subtracted from each value for AMT or MTX treated mice. Mean tissue content of AMT (*triangles*) and MTX (*circles*) are shown; *error bars* indicate standard error of the mean. Only in the brain were there significant differences between the AMT and MTX content at each time point

extracellular concentrations, we show that steady-state AMT tissue concentrations were similar to those of MTX, despite a presumed shorter average polyglutamate chain length.

We did not measure FPGS activity in the murine tissues studied. However, tissue-specific variation in the FPGS expression in mice [14, 44] and humans [27, 45] may be responsible for this observed difference in an-



c DHFR IC_{50} (nM)

	parent	-glu$_2$	-glu$_3$	-glu$_4$	-glu$_5$
MTX	3.32	3.83	1.87	1.63	1.44
AMT	4.12	3.53	not tested		

Fig. 3 DHFR inhibition by MTX and AMT polyglutamates in vitro. Purified recombinant DHFR was incubated with varying concentrations of antifolate or antifolyl polyglutamate, and enzyme activity was measured by spectrophotometrically observing the conversion of NADPH to NADP. Each concentration of inhibitor was tested at least in triplicate. Mean ± SEM DHFR activity in the presence of MTX (**a**) or AMT (**b**) and the respective polyglutamate species are shown relative to uninhibited DHFR activity (control). The concentration of each compound found to inhibit activity by 50% (IC_{50}) is shown in **c**

tifolate accumulation between the CNS and peripheral organs. In both mice and humans, the greatest expression of FPGS is seen in the liver, followed by the kidney, and then the spleen, while the brain has a low to undetectable FPGS message. This ranking generally parallels the observed total organ-specific antifolate accumulation we observed (see the *y*-axis in Fig. 1). One might expect that, under conditions of very low FPGS expression, as in the brain, an antifolate with greater affinity for FPGS (AMT) would be preferentially accumulated relative to the one with lower affinity (MTX), but this is not what we observed.

It is possible that the decreased accumulation of AMT by the brain parenchyma will result in a decrease in the frequency or severity of subacute or delayed antifolate-related neurotoxicity, a hypothesis we are now testing in the clinic. If validated, the potential for causing decreased neurotoxicity with equivalent peripheral efficacy would suggest a greater therapeutic index for AMT relative to MTX.

In this regard, we do not expect that lower CSF concentrations of AMT will result in an increased CNS relapse rate among patients with ALL, however, because all patients with ALL on our protocol received prophylactic triple intrathecal therapy, and the CSF concentration of MTX immediately following IT administration (>100 μm) [5] is many orders of magnitude greater than that seen after systemic standard-dose therapy (~20 nM). Moreover, with approximately 2 years of median follow-up there have been no CNS relapses among the 29 patients with ALL treated on CINJALL with AMT [7]. In addition, the observation that AMT and MTX concentrations within the testicular tissue are similar suggests that the substitution of AMT for MTX in leukemia therapy would not result in excess rates of testicular relapse either.

In summary, we have previously shown AMT to have greater clinical potency than MTX, with complete oral bioavailability [33], and greater accumulation and metabolism by leukemic blasts [43]. We now show decreased penetration into the brain parenchyma, relative to MTX, at doses that produce equivalent penetration into hematopoietic tissues (blood, liver, and spleen) as well as other peripheral organs (kidney, and testes). This sparing of the CNS may result in decreased neurotoxicity, and provides further support for the more complete testing of AMT as a single agent in rheumatological diseases (in which up to half of the patients treated with MTX experience CNS toxicity severe enough to endanger compliance) [4] and in multi-agent therapy for patients with ALL (in whom the use of high-dose MTX in conjunction with intrathecal chemotherapy is associated with neurotoxicity) [28].

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