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ORIGINAL ARTICLE

Phase 2B trial of aminopterin in multiagent therapy for children with newly diagnosed acute lymphoblastic leukemia

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Abstract
Purpose Aminopterin offers advantages over the related antifolate, methotrexate, including greater potency, complete bioavailability, and more consistent accumulation and metabolism by patients' blasts. This current trial was done to document the toxicity of the aminopterin within a multiagent therapeutic regimen for children with newly diagnosed ALL.
Experimental Design Patients at high risk of relapse were non-randomly assigned to therapy including oral aminopterin 4 mg/m^2, in two doses 12 h apart, in place of methotrexate 100 mg/m^2 in four divided doses.
Results Thirty-two patients, 22 with pre-B ALL and ten with T-lineage ALL, have been treated with aminopterin, with median follow up of 40 months. Hematologic, mucosal and hepatic toxicity has been tolerable and reversible. There have been no toxic deaths among patients in remission. During weekly AMT therapy, higher mean neutrophil counts were observed among patients who were wild type for polymorphisms in methylene tetrahydrofolate reductase and methionine synthase reductase.
Conclusions Aminopterin can be safely incorporated in multiagent therapy for patients with ALL, in place of systemic methotrexate, without causing excessive toxicity. These results support a larger trial comparing the efficacy and toxicity of aminopterin and methotrexate in therapy for patients with ALL.

Keywords Aminopterin · Methotrexate · Acute lymphoblastic leukemia · Antifolate · Therapeutic trial

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Introduction

The folate antagonist methotrexate (4-amino-4-deoxy-10-*N*-methyl-pteroylglutamic acid; MTX) has been a central component of therapy for patients with acute lymphoblastic leukemia (ALL) for more than five decades. However, the persistence of both clinical resistance to MTX and MTX-induced toxicity prompts continued interest in expanding the antifolate armamentarium beyond this single folate analog to others with altered pharmacodynamic properties. One strategy involves the clinical development of antifolates with enhanced membrane transport and polyglutamation in tumor cells, which results in increased intracellular accumulation and enhanced cytotoxicity. Edatrexate [49], pralatrexate [37] and aminopterin (4-amino-4-deoxy-pteroylglutamic acid; NSC 739; AMT) [53] are three antifolate analogs that exhibit these properties in vitro [50, 51].

Of these three, we have focused on the clinical development of AMT, an antifolate whose structure more closely resembles the vitamin folic acid than either edatrexate or pralatrexate. Like pralatrexate, AMT has greater affinity than MTX for folylpolyglutamate synthetase (FPGS) [14, 34], the cytoplasmic enzyme that adds glutamate residues to folates and classical antifolates, enhancing cellular retention. As a result, increased accumulation and metabolism of AMT relative to MTX can be demonstrated by patients' leukemic blasts in vitro [5, 53]. AMT also has nearly three-fold greater potency than pralatrexate at the primary intracellular target dihydrofolate reductase (DHFR) [27, 37, 51] and more complete oral bioavailability than has been reported for MTX [23]. In addition, because AMT is more potent than MTX, it can be given at lower doses, leading to decreased accumulation of AMT in the CNS [6]. When combined with adequate prophylactic intrathecal therapy to prevent CNS relapse, this property may result in less neurotoxicity and, consequently, an improved therapeutic index for AMT relative to MTX. These possible therapeutic and safety advantages of AMT relative to MTX remain to be tested clinically.

Although AMT had been the first chemotherapy drug to produce remissions among children with ALL [12], it was replaced in the clinic by MTX because MTX was felt to have a superior therapeutic index [9, 16, 32, 33]. Consistent with its greater uptake and potency at DHFR, our Phase I trial [40] identified a maximal tolerated dose (MTD) for AMT (4 mg/m^2/week in two doses 12 h apart) that was 7- to 15-fold lower than the MTD of pralatrexate [26] and 25-fold lower than the MTX dose on a comparable divided-dose regimen [58]. The phase II trial in patients with refractory leukemia confirmed complete oral bioavailability of AMT and showed that oral AMT at this dose and schedule had activity among children with refractory ALL [5].

This current study was conducted to pilot the substitution of AMT at its MTD for oral MTX within multiagent therapy, including an intensive Capizzi-style antifolate-asparaginase combination [4] given sixteen times over 32 weeks. The data presented here demonstrate that this AMT-containing regimen did not lead to excessive toxicity or early relapse rates among the 32 patients treated, and support a larger trial to examine the relative efficacy and safety of AMT versus MTX.

Materials and methods

Materials

AMT tablets were supplied by the Cancer Institute of New Jersey (CINJ). Synthesis of AMT tablets was supported in part by an FDA Orphan Products Development grant (FD-R-001832-03). AMT was synthesized per the previously reported method of Piper and Montgomery [38] and the powder pressed into a 1 mg scored tablet by the University of Iowa under IND #49,927. Purity of the AMT tablet was assayed at CINJ using isocratic elution on a reverse-phase C18 column, with PDA spectrophotometric detection between 190 and 400 nm. Comparison of the retention time and absorption spectra of individual peaks with known standards showed the AMT tablets to be over 98% pure AMT. Folic acid accounts for less than 0.3% of the total, with breakdown products such as pterins and PABA accounting for the remainder [5].

Clinical trial

The Cancer Institute of New Jersey Acute Lymphoblastic Leukemia trial (CINJALL) was designed to pilot the substitution of AMT for MTX within the setting of multi-agent therapy for children with newly diagnosed ALL at high risk of relapse. The protocol was approved by the Institutional Review Board (IRB) of the University of Medicine and Dentistry of New Jersey, and opened in March, 2001. Informed consent was obtained from each subject or subject's guardian.

The treatment schema was previously published [6], and is shown in Table 1. Children with ALL at standard risk (SR) of relapse by NCI criteria (age 1–10 years, WBC < 50,000/μl, no CNS or testicular disease, and no unfavorable cytogenetics) were treated with multiagent chemotherapy, including divided-dose oral MTX as previously published, 25 mg/m^2/dose for four doses 6 h apart [58]. Those at high risk (HR) of relapse (all others) were non-randomly assigned to receive a single Delayed Intensification and oral AMT at its MTD, 4 mg/m^2 per week in two doses 12 h apart [40], in place of oral MTX. Sixteen doses of asparaginase are given over the first 32 weeks of Intensive Continuation, after MTX or AMT [4]. Patients

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

Induction (4 weeks)	Daunomycin	60 mg/m^2 IV day 1
	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)
	L-asparaginase	10,000 units/m^2 IM days 2,8,11,15,18,22
	IT Triples	Days 1, 15, 29
Consolidation (12 weeks)	Aminopterin	2 mg/m^2/dose po q12 h for 2 doses each week.
	Leucovorin	5 mg/m^2 × 1 dose, 48 h after 1st AMT, weeks 2, 4, 6, 8, 10, 12
	IT Triples	Triple intrathecal therapy weeks 3, 5, 7, 9, and 11
	6-Mercaptopurine	37.5 mg/m^2/dose po twice daily
Delayed intensification (8 weeks)	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 d1, 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	1000 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)	Aminopterin	2 mg/m^2/dose po q12 h for 2 doses, weeks 1,3,5,7
	Leucovorin	5 mg/m^2 × 1 dose, 48 h after first AMT
	6-Mercaptopurine	37.5 mg/m^2/dose po twice daily
	L-asparaginase	10,000 IU/m^2 IM < 6 h after 2nd AMT 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)
	Dexamethasone	3 mg/m^2 po twice daily × 7 days, starting day 1, week 8
	IT Triples	Week 8 of each cycle + week 3 of the first four cycles
Continuation (until 30 months post remission)	Aminopterin	2 mg/m^2/dose po once, weeks 1–7
	6-Mercaptopurine	37.5 mg/m^2/dose po twice daily
	IT Triples	Week 8 of each cycle

IT Triples refers to the combination of methotrexate, cytarabine and hydrocortisone, dosed by age and given intrathecally

with overt CNS leukemia were given craniospinal radiation after cycle four of intensive continuation, and no further intrathecal chemotherapy.

Patients with T-lineage disease were not excluded, based on the following observations: (1) the inclusion of intensive MTX/asparaginase-based antimetabolite therapy improves outcome for patients with T-ALL [1, 15]; (2) AMT is more consistently metabolized to polyglutamates by patients' T-ALL lymphoblasts than MTX [5]; and (3) single agent AMT produced CRs among patients with refractory T-ALL on our Phase II trial [5].

Stopping rules were designed to detect an excess in either toxicity or early relapse among those patients receiving AMT compared to those on the MTX arm and historical controls. Based on the historical data (the identical protocol, with the exception of methotrexate in place of aminopterin), we defined the unacceptable rate of early relapse and unacceptable rate of changing back to methotrexate for severe aminopterin-related toxicity at 15%. Using binomial distribution, a table was generated of the posterior probability that the observed proportion of patients with early relapse or toxicity exceeds 15%, for $n = 3$–300 patients. Interim toxicity and response data were monitored quarterly by the Cancer Institute of New Jersey Human Research Oversight Committee, biannually by an external committee, and annually by the FDA. The stopping rule would apply at any time that the posterior probability that $P > 15\%$ was greater than 90%.

Protocol amendment adding leucovorin rescue

Leucovorin rescue was not prescribed following AMT in the initial study design, with the rationale that the absolute dose of AMT is low relative to the recommended daily allowance for folate and that plasma folate is higher now in the era of FDA-mandated folate supplementation of food [21] than it was when neurotoxicity was observed in patients treated with oral divided-dose MTX [57]. However, two of the first five HR patients experienced CTC grade 3 neurotoxicity during consolidation (weekly oral antifolate, twice daily mercaptopurine, and triple intrathecal therapy every other week; Table 1). A detailed description of

these events is presented in the results. The protocol was subsequently amended, in February 2002, to add a single dose of oral leucovorin, 5 mg/m^2, 48 h after the first dose of AMT on weeks that intrathecal chemotherapy is not given. This dose amounts to half the leucovorin given to patients on the MTX arm (10 mg/m^2, divided in two doses, 12 h apart).

Analysis of clinical toxicity

All enrolled patients treated with AMT were eligible for analysis of toxicity. Patients were assessed for toxicity at each encounter and were instructed to report any suspected toxicity to the investigators. Blood counts were measured at least weekly during consolidation, every other week during intensive continuation, and monthly during continuation therapy. Chemistries including BUN, creatinine, SGOT, SGPT, and total bilirubin were measured at least every other week during consolidation and every eight weeks during intensive continuation and continuation. When total bilirubin exceeded 2 mg/dl, direct bilirubin was measured. All toxicity was graded on a scale of 0–5, using the National Cancer Institute Common Terminology Criteria (CTC) version 3.0. All toxicities occurring after any administration of AMT were reported to the study coordinator and followed to the end of the study or until resolution. All unexpected toxicity or toxicity that resulted in hospital admission was reviewed by the study coordinator and reported as a Serious Adverse Event (SAE) to the IRB. The toxicity analyses of SAEs experienced by the 32 patients who received AMT, focused on the toxicities that might be attributable to AMT (i.e. excluding toxicity during induction and delayed intensification, where AMT is not given).

Pharmacokinetics

To confirm oral bioavailability of AMT, limited sampling pharmacokinetic analysis was conducted, as previously described [40]. Briefly, two ml of blood were collected in an EDTA tube prior to, and 0.5, 1, 2, 3, 5, and 12 h after the AMT was administered. AMT concentrations in plasma were determined using a radioligand-binding assay, as previously published [5, 40]. Noncompartmental analysis was done to calculate the AUC, using the linear trapezoidal rule, and extrapolated to infinity.

Red blood cell (RBC) MTX and AMT

RBC AMT was measured to monitor compliance and to estimate antifolate exposure to the bone marrow [48]. Samples were collected for analysis at the end of each 8–12 week treatment cycle, because RBC antifolate reaches steady-state after a minimum of 4–6 weeks of unchanged oral therapy [19]. RBC AMT was assayed as previously described [6], using radioligand binding assays. RBC AMT concentrations were compared with RBC MTX concentrations from concurrent standard risk patients as well as historical controls analyzed over two decades by the same techniques in the same laboratory [17].

Analysis of single nucleotide polymorphisms

All patients were screened for the presence of functional polymorphisms in the following genes related to folate and antifolate metabolism: methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C, aminoimidazole carboxamide ribonucleotide transformylase (ATIC) C347G, γ-glutamyl hydrolase (GGH) C452T, methionine synthase (MS) A2756G, and methionine synthase reductase (MTRR) A66G. DNA was isolated from mononuclear cells in bone marrow or peripheral blood, using the QiAmp DNA blood kits (QIAGEN, Valencia CA). Genotypes were determined using TaqMan-based allelic discrimination assays and pre-developed probe/primer sets (Applied Biosystems, Foster City, CA). PCR amplification using 5 ng/sample of genomic DNA was done in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems) with an initial step of 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescence profile of each well was measured in an Applied Biosystems 7900HT Sequence Detection System and the results analyzed with Sequence Detection Software (Applied Biosystems).

Results

Patients

Between March 2001 and September 2005, 58 children or young adults with ALL enrolled on CINJALL: 21 at standard risk (SR) of relapse, treated with MTX, and 37 at high risk (HR) of relapse. The median age of the HR patients treated with AMT was 14 years (range 2–20).

HR patients with T-lineage disease had higher initial WBC counts (169.9 ± 50.70; $N = 10$) than those with B lineage disease (40.95 ± 23.6; $N = 22$; $P = 0.01$, two-tailed t-test). They also tended to be more likely to have blasts in their initial CSF samples (3 of 10 with T-lineage disease versus 1 of 22 with B-lineage disease; $P = 0.08$, Fisher's exact test) and to be male (7 of 10 vs. 9 of 22; $P = 0.11$). There was no significant difference in age at presentation between those with B-lineage (12.7 ± 1.1 years) and those with T-lineage ALL (13.3 ± 1.7 years; $P > 0.5$).

Five HR patients did not receive AMT. Two patients elected to go off protocol for alternative therapy at another institution within four weeks of initial diagnosis. Two died

in induction due to infection-related complications. There was one induction failure, a 23-year-old man with Philadelphia chromosome positive ALL, who subsequently died of leukemia-related complications. The characteristics of the remaining 32 patients who entered remission and were treated with AMT are shown in Table 2. At the time of data analysis, June 2007, the median follow-up for this cohort was 172 weeks, and 22 had completed all scheduled therapy (Table 2). There have been six relapses and one secondary AML (22% of the 32 patients treated with AMT; Table 2). Three patients remain in the final phase of therapy, continuation, receiving weekly low-dose oral AMT. The last patient will complete continuation in February 2008.

Toxicity

Accidental AMT overdose

One patient misunderstood her discharge instructions during Consolidation and took AMT twice a day for 6 days, rather than a total of two doses, 12 h apart. She presented

Table 2 Characteristics of patients treated with AMT

Age (years)	Sex	Race	Phenotype	Initial WBC ($\times 10^3/\mu l$)	CNS	Disease outcome/current status	CR Duration (months)
17	M	W	T	179	1	Completed Tx; CCR	75.9
17	M	W	B	10.9	1	Completed Tx; CCR	75.2
16	M	W	T	70.6	3	Completed Tx; CCR	74.3
14	F	PI	B	3.8	1	Completed Tx; CCR	3.1
11	F	H	B	8.3	1	Completed Tx; CCR	71.5
12	F	A	B	4	1	Completed Tx; CCR	66.1
5	F	>1	B	59	1	Secondary AML; died due to AML	30.9
14	M	W	B	2.6	1	Completed Tx; CCR	59.7
2	M	A	T	340	1	CNS/BM Relapse; died due to 2nd relapse of ALL after BMT	40.7
17	M	H	B	4.1	1	Completed Tx; CCR	58.3
18	M	W	B	15.4	1	Completed Tx; CCR	55.6
14	F	W	B	7.4	1	Completed Tx; CCR	55.2
13	M	W	B	2.3	1	Completed Tx; CCR	55.0
4	F	>1	B	522	1	Completed Tx; CCR	54.5
13	F	W	B	3.7	1	BM Relapse; died in re-induction	15.6
3	F	H	B	97.4	1	Completed Tx; CCR	51.5
19	F	H	B	6.8	1	Philadelphia chromosome positive; died of BMT-related complications, in remission	6.7
15	F	H	B	0.6	1	Completed Tx; CCR	44.2
16	F	W	T	48.7	1	BM Relapse; died in reinduction	17.2
4	F	W	B	73.3	1	Completed Tx; CCR	43.6
2	M	W	B	18.3	3	Completed Tx; CCR	42.5
14	F	W	B	4.5	1	Completed Tx; CCR	39.7
15	F	W	B	40	1	BM Relapse; died due to ALL	22.9
19	M	W	B	6.6	1	Completed Tx; CCR	35.9
14	M	W	B	2.5	1	Completed Tx; CCR	35.0
19	F	A	T	113.6	1	Completed Tx; CCR	35.0
18	M	W	T	170	1	Completed Tx; CCR	33.1
9	F	W	T	549.9	3	CNS Relapse; died due to ALL	9.6
10	M	H	T	67.4	1	On Tx; CCR	26.2
20	F	H	B	7.4	1	BM relapse; Declined further treatment	23.9
11	M	W	T	121.8	1	On Tx; CCR	23.7
15	M	W	T	38.2	2	On Tx; CCR	22.5

Abbreviations: W, white non-Hispanic; H, Hispanic; PI, Pacific Islander; A, Asian; >1, identified as of more than one racial/ethnic group; CCR, Continuous complete remission; B, B-precursor ALL; T, T-lineage ALL

with the expected toxicity resulting from antifolate overdose, including grade 4 mucositis, pancytopenia, fever, and generalized erythroderma. After intensive supportive care and leucovorin rescue, she recovered over 10 days in the hospital. Her liver function tests remained normal during this episode. Per protocol guidelines, she resumed AMT when her neutrophil count was over 500/μl and platelets were over 75,000/μl. This patient, with Philadelphia chromosome positive ALL, subsequently elected to undergo matched sibling stem cell transplantation, but died in remission of transplant-related toxicity.

Laboratory abnormalities

The frequency of CTC grade 3–4 hematologic, hepatic, or renal toxicity is shown in Table 3. Neutropenia was the most common toxicity in each phase of therapy, and all patients experienced grade 3–4 leucopenia and neutropenia on at least one occasion. Elevation of serum transaminases above the upper limit of normal also occurred for all patients at some point during therapy, although only 10 patients (31%) experienced grade 3–4 SGPT elevation. In all cases, hepatic dysfunction during and following CINJ-ALL therapy was transient and reversed without holding therapy. Nephrotoxicity, as measured by increases in serum blood urea nitrogen or creatinine was uncommon and mild. No patients experienced toxicity ≥grade 2 at any phase of post-remission therapy.

Anemia was related to AMT dose intensity. Average hemoglobin concentrations were lowest during consolidation (mean ± SEM 10.2 ± 0.2 g/dl), higher in intensive continuation (11.5 ± 0.2 g/dl), and highest during continuation (13.3 ± 0.4 g/dl; $P < 0.001$ for each comparison, two-tailed *t*-test).

Effect of concurrent therapy on toxicity

In addition to the effect of AMT dose intensity, the hematologic toxicity of AMT was modulated by other concurrently administered antileukemia therapy. Each patient's mean ANC was lower, for example, during the first four intensive continuation cycles (including asparaginase every other week) than in the subsequent four cycles (1,840 ± 130 cells/μl vs. 2,730 ± 150 cells/μl; $n = 30$; $P < 0.0001$, two-tailed paired *t*-test) and the frequency of grade 3–4 neutropenia decreased from 10% of CBCs to 3% ($P = 0.002$; two tailed paired *t*-test). The seven-day dexamethasone pulses during week 8 of each intensive continuation cycle were followed by significantly higher average ANCs (4,218 ± 218 cells/μl) than were observed in the week prior to the pulses (1,494 ± 82 cells/μl; $n = 30$; $P < 0.001$, two-tailed paired *t*-test). Dexamethasone and asparaginase had no corresponding effects on other laboratory markers of toxicity.

Fever and Infections

In each phase of therapy, the most common serious adverse events were infectious. Fifty-six events occurred, including 8 documented infections (bacteremia [$n = 2$], urinary tract infections [$n = 2$], pneumonia, acute otitis media, sinusitis, and zoster) or unexplained fevers with ($n = 29$) or without ($n = 19$) neutropenia. There were no infectious deaths in remission.

Gastrointestinal toxicity

Twenty-nine SAEs described gastrointestinal toxicity during treatment phases that included AMT. One patient developed pancreatitis during intensive continuation, after

Table 3 Laboratory parameters during therapy with AMT

Grade 3–4 toxicity	Consolidation AMT 4/week $n = 32$	Intensive Continuation AMT 4 qow $n = 31$	Continuation AMT 2/week $n = 26$	Any Phase
WBC < 2,000/μl	24 (75%)	28 (90%)	10 (38%)	32 (100%)
ANC < 1,000/μl	28 (88%)	29 (94%)	14 (52%)	32 (100%)
Hgb < 8 g/dl	13 (41%)	12 (39%)	2 (8%)	22 (69%)
Platelets < 50,000/μl	7 (22%)	12 (39%)	3 (12%)	18 (56%)
SGOT > 200 u/dl	2 (6%)	2 (6%)	0 (0%)	3 (9%)
SGPT > 200 u/dl	8 (25%)	6 (19%)	1 (4%)	10 (31%)
BUN or Creat	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Mucositis	4 (13%)	0 (0%)	0 (0%)	4 (13%)

AMT was given at a dose of 4 mg/m^2/week in Consolidation, 4 mg/m^2 every other week (qow) in Intensive Continuation, and 2 mg/m^2/week in Continuation. Blood counts were measured at least weekly during consolidation, every other week during intensive continuation, and monthly during continuation therapy. Chemistries including BUN, creatinine, SGOT, SGPT, and total bilirubin were measured at least every other week during consolidation and every eight weeks during intensive continuation and continuation. The number (and percentage) patients who demonstrated evidence of grade 3 or 4 toxicity at one or more determinations is shown by phase of AMT therapy

administration of asparaginase. There were 21 instances of diffuse abdominal discomfort, anorexia, nausea, and/or vomiting among nine of the 32 patients. One patient was admitted to the hospital with these complaints on seven separate occasions.

Stomatitis was infrequent. A single patient developed grade 4 mucositis, after an accidental AMT overdose (2 mg/m^2 twice a day for 6 days, rather than a total of two doses, 12 h apart). Three patients (9%) experienced grade 3 mucositis during Consolidation (intensive weekly AMT therapy with triple intrathecal therapy every other week), including one patient who was admitted with mucositis on three occasions. Two additional SAEs described grade 2 mucositis: one each during Consolidation and Intensive Continuation.

Neurologic toxicity

Two of the first five patients experienced grade III neurotoxicity during consolidation (one of them had this toxicity on two separate occasions), prior to the protocol amendment adding leucovorin rescue on weeks intrathecal therapy is not given. Between seven and ten days after triple intrathecal chemotherapy (including 15 mg MTX), both patients experienced symptoms typical of subacute neurotoxicity associated with intrathecal MTX [39, 56], including dysarthria and hemiplegia. One of these two had a positive family history for vascular disease with early death in two grandparents and was found to have an anatomical narrowing of the middle cerebral artery. Per protocol design, oral MTX was substituted for AMT for this patient, and extra leucovorin rescue was given. Both patients recovered completely within seven days, and have been in continuous complete remission for more than 5 years.

Subsequent to the protocol amendment (see Methods), two additional patients have presented with unilateral weakness and cranial nerve palsies (Grade 3 toxicity) within two weeks after intrathecal MTX. Both cases occurred during Delayed Intensification, a phase in which no AMT is given (Table 1). In neither case was intracranial pathology found on imaging studies (CT or MRI of the head). Both patients were treated with dextromethorphan, 2 mg/kg/dose every 12 h [11], and experienced complete resolution of symptoms within 24 h.

Additional neurologic symptoms included headaches (seven events among four patients), limb pain ($n = 3$) related to vincristine or osteonecrosis, and near-syncope associated with dehydration ($n = 1$).

Bone toxicity

Three HR patients developed severe hip or knee pain during the course of therapy, with radiographic evidence of avascular necrosis (AVN). This incidence of AVN (3 of the 15 HR patients aged 14 or older who have completed at least one year of therapy, 20%) is similar to that published for teenagers on cooperative group leukemia trials [31, 54] and is most likely attributable to dexamethasone.

Effect of genetic polymorphisms on AMT toxicity

All 58 enrolled patients were tested for the presence of SNPs in MTHFR, MTRR, ATIC, MS, and GGH (Table 4). For each SNP, the observed variant allele frequencies in this population are similar to published frequencies in larger populations [55]. There was no significant difference in gene frequencies between those patients treated on the HR and SR arms (data not shown).

Only two of these genes were related to hematologic toxicity during weekly AMT therapy. HR patients who had one or more variant MTHFR alleles ($N = 22$) had significantly lower average neutrophil counts during weekly AMT therapy (1,613 ± 84 neutrophils/μl) than those who are homozygous wild-type at both MTHFR 677 and 1298 (2,687 ± 333 neutrophils/μl; $N = 7$; $P < 0.001$, two-tailed unpaired t-test). HR patients homozygous for the variant MTRR allele 66A had lower average neutrophil counts (1,195 ± 109/μl; $N = 6$) than those who were heterozygous or homozygous wild-type (2,050 ± 142; $N = 23$; $P < 0.01$). There were no significant relationships between genotype and frequency or severity of hepatic, mucosal, or neurologic toxicity.

Table 4 Frequency of single nucleotide polymorphisms (SNPs) among patients on CINJALL

	MTHFR 677 C > T	MTHFR 1298 A > C	ATIC 347 C > G	MS 2756 A > G	MTRR 66 G > A	GGH 452 C > T
Homozygous wild type	25 (43%)	33 (57%)	28 (48%)	38 (66%)	18 (31%)	48 (83%)
Heterozygous	29 (50%)	19 (33%)	20 (34%)	19 (33%)	30 (52%)	10 (17%)
Homozygous for the variant allele	4 (7%)	6 (10%)	10 (17%)	1 (2%)	10 (17%)	0 (0%)
Variant allele frequency (%)	32	27	34	18	43	9

DNA from all enrolled patients was analyzed for the presence of SNPs in methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C, aminoimidazole carboxamide ribonucleotide transformylase (ATIC) C347G, γ-glutamyl hydrolase (GGH) C452T, methionine synthase (MS) A2756G, and methionine synthase reductase (MTRR) A66G

Pharmacokinetics

Eighteen patients consented to have samples collected for analysis of AMT pharmacokinetics. The mean peak plasma concentration (±standard error) was 0.26 ± 0.02 μM. The mean calculated volume of distribution was 0.59 ± 0.09 l/kg. The mean AUC after oral AMT, at a dose of 2 mg/m^2, was 1.06 ± 0.07 μmol h/l. This value is identical to the AUC after administration of the oral liquid on the Phase I trial (1.05 ± 0.07 μmol h/l; $n = 13$) [40], suggesting that concurrent administration of mercaptopurine does not alter the pharmacokinetics of oral AMT. The mean plasma half-life for AMT was 4.3 ± 0.46 h.

Two patients had poor oral absorption of AMT (AUC < 0.2 μmol h/l) when first studied. One, a toddler with Down syndrome, was given AMT crushed and mixed in a small volume (<1 ml) and may not have swallowed the dose appropriately. The other, a teenager in the intensive care unit with a large pleural effusion, was receiving parenteral nutrition and may have had poor gastrointestinal motility. When restudied, both had normal kinetics, with an AUC within one standard deviation of the group mean (0.81 and 0.73 μmol h/l, respectively). In addition, at the end of consolidation, both patients had steady-state RBC AMT concentrations within one standard deviation of the mean, suggesting adequate absorption of AMT over the course of the 12-week treatment phase.

Bone marrow penetration by AMT, as indicated by RBC AMT concentrations

As previously published [6], mean RBC AMT (277 ± 38 pmol/ml RBC; $n = 16$) at the end of 12 weeks of oral AMT therapy (4 mg/m^2/week), was not statistically different from mean RBC MTX after 12 weeks of oral MTX (100 mg/m^2/week) among concurrent SR patients ($n = 13$) or historical patients treated with the same regimen ($n = 79$; 271 ± 63 pmol/ml RBC for the combined groups). Bone marrow exposure to AMT, as indicated by RBC AMT accumulation, appears to be proportional to the weekly dose. RBC AMT after cycles where patients were given oral AMT 2 mg/m^2/week or 4 mg/m^2 every other week were approximately half that seen among patients given 4 mg/m^2/week (Fig. 1).

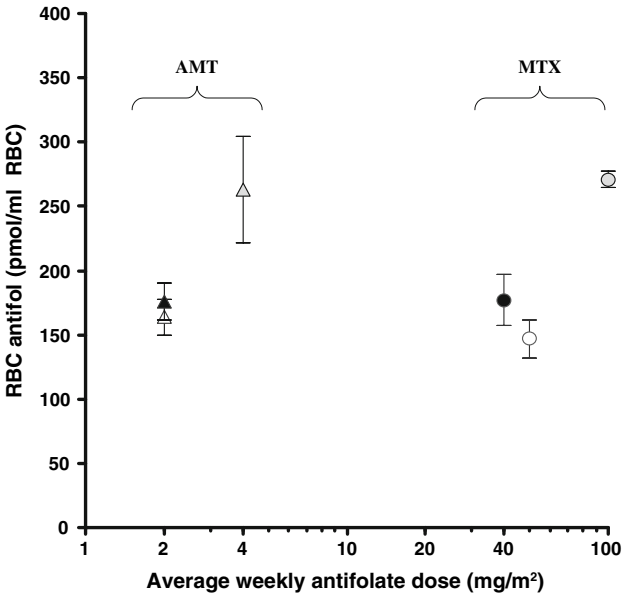

Fig. 1 Steady-state RBC antifolate concentrations. RBC AMT concentrations (an indirect indicator of bone marrow exposure) were measured after at least 8 weeks of antifolate dosing. For comparison, RBC MTX is shown from concurrent standard risk patients as well as historical controls analyzed over two decades by the same techniques in the same laboratory. AMT was given at a dose of 4 mg/m^2/week in Consolidation (shaded triangle; n = 17), 4 mg/m^2 every other week in Intensive Continuation (open triangle; n = 56), or 2 mg/m^2/week in Continuation (solid triangle; n = 44). MTX was given at a dose of 100 mg/m^2/week in Consolidation (shaded circle; n = 91), 100 mg/m^2 every other week in Intensive Continuation (open circle; n = 50), or 40 mg/m^2/week in Continuation (solid circle; n = 28). RBC AMT is proportional to the average weekly dose. AMT is 20–25 fold more potent than MTX

Discussion

Intensification of antimetabolite therapy has been credited for the steadily increasing cure rates for children with ALL [36] as well as for the superior outcome of adolescents and young adults with ALL treated on pediatric versus adult protocols [35]. With the goal of intensifying antimetabolite therapy, MTX may be given intravenously at high doses (i.e. individual doses ≥ 500 mg/m^2/dose), an approach currently being explored in cooperative group trials. Alternatively, intensification of antifolate therapy can be achieved by increasing the time of exposure, rather than the magnitude of each dose. Prolonged administration of weekly oral MTX at lower individual doses (25–50 mg/m^2/dose) results in similar event-free survival [29, 30, 58], but with fewer inpatient days [29] and less acute neurotoxicity [28].

Earlier versions of the treatment protocol reported here employed this time-intensive strategy, capitalizing on the observation that the cytotoxic effects of MTX and AMT are more dependent on time above a threshold dose than on peak concentration [52]. The backbone of the original regimen had its basis in seminal work by Capizzi [4], and included 28 courses of divided-dose MTX over 18 months, with asparaginase given 2–4 h after MTX [58]. The successor protocol further augmented therapy by extending MTX therapy to 44 courses in the same time period, in the setting of twice-daily mercaptopurine. Because AMT possesses potential pharmacodynamic advantages over MTX, we pursued a divided weekly AMT dosing strategy. Further

supported by the clinical responses and tolerable toxicity on the Phase I and II trials [5, 40], the clinical trial described here was designed to test the toxicity associated with substituting AMT for MTX in the context of multiagent therapy for children with ALL at high risk of relapse.

Antifolate therapy was non-randomly assigned on this treatment protocol. Therefore, we do not have a statistical basis to compare the toxicity observed among patients on the SR and HR treatment arms, and a larger, randomized comparison of AMT and MTX will be necessary to contrast their toxicity profiles. However, we expected the patients on the HR arm of this trial to experience greater hematologic, infectious, mucosal, orthopedic and hepatic toxicity than the SR patients, because of their older age and more advanced disease [46]. In contrast with this expectation, and with reports from the early history of antifolate use indicating that the toxicity of AMT was prohibitive [9, 32, 33], we did not see excessive or intolerable toxicity in our patients treated with AMT.

It is tempting to compare the toxicity experienced by this cohort with that described for patients treated with MTX on an earlier version of this protocol using intensive oral divided-dose MTX (the Dallas-Fort Worth protocol; DFW) [58] or POG 9005, which tested oral divided dose MTX [29]. However, such a comparison is confounded by differences in the patient population (e.g. inclusion of patients with T-lineage ALL on this protocol) and changes in protocol therapy other than the substitution of AMT for MTX (e.g. reduction of asparaginase doses in intensive continuation from 32 in DFW to 16; different MTX schedule and greater leucovorin rescue on POG9005). Nevertheless, the toxicity experienced by these HR patients receiving AMT appears to compare favorably. For example, transaminase elevations at least five times the upper limit of normal occurred in 59% of 239 patients treated with oral MTX on the DFW protocol [13], compared with only 28% of HR patients on this trial treated with AMT. Hospital admission for febrile neutropenia complicated 2.5% of the MTX-asparaginase courses administered to patients on DFW [58], compared to 29 among our patients who have collectively received 1,220 courses of divided dose AMT (2.4%). Grade 3–4 mucositis occurred at similar frequencies among the patients treated with oral MTX on POG9005 (28 of 350; 8%) [29] or this protocol (4 of 32; 12.5%).

It is possible that AMT will cause less neurotoxicity than MTX. MTX-induced neurotoxicity is most common following high-dose intravenous administration [22, 28, 41], but can occur among patients receiving low-dose oral MTX as well [2, 42]. Uptake of AMT and MTX through the blood-brain barrier is believed to be concentration dependent, with CSF concentrations reaching 1–2% of serum. Because AMT is more potent than MTX [8, 47], it is used at 1/25 the dose, resulting in lower concentrations in the brain and CSF [6]. As a result of these pharmacodynamic differences between AMT and MTX, AMT may therefore cause less neurotoxicity when used at a dose expected to have equivalent peripheral effects to MTX.

Two patients treated with AMT on this trial experienced neurotoxic events prior to the protocol amendment adding leucovorin rescue. However, in both cases, the timing and clinical presentations were consistent with subacute MTX-induced neurotoxicity caused by intrathecal MTX [39, 56]. Furthermore, the low nanomolar CSF AMT concentrations after oral dosing [6] are many orders of magnitude below the concentration of MTX after intrathecal administration (>100 micromolar) [3]. No similar neurotoxicity was observed among patients treated with AMT alone, at the same dose, on the Phase II trial [5]. Nevertheless, a contribution by AMT to neurotoxicity can not be excluded. Prospective evaluation of neurocognitive function is ongoing for this cohort, and will be of critical importance in any trials evaluating changes in antifolate drug or dosing.

While decreased CNS penetration may be beneficial in terms of limiting neurotoxicity, use of AMT might result in an increased rate of CNS relapse. Within the context of repeated triple intrathecal prophylaxis (at least 24 administrations of intrathecal methotrexate, cytarabine and hydrocortisone after remission induction; Table 1), we observed two instances of CNS relapse among the 32 patients treated with AMT (Table 2). Both of these patients had T-lineage disease: one patient had CNS disease at diagnosis and an isolated CNS relapse after 11 months of treatment, and another with hyperleukocytosis at diagnosis had a combined CNS/BM relapse one year after the completion of therapy.

A phase III trial is necessary to define the rate of extramedullary relapse among patients treated with systemic AMT. The use of intrathecal AMT has been explored in the past [18, 43], and could be considered in a clinical trial comparing AMT with MTX. The finding that AMT and MTX penetration of testicular tissue is similar [6] suggests that the substitution of AMT for MTX in leukemia therapy will not result in excess rates of testicular relapse.

As has been observed for MTX [7, 10, 25], we note relationships between AMT toxicity and SNPs in genes relevant to folate metabolism. In this small cohort of patients treated with AMT, hematologic toxicity varied with MTHFR and MTRR SNPs. The enzyme MTHFR is critical to maintaining physiologic intracellular concentrations of reduced folates. The presence of functional polymorphisms in this gene has been associated with an increase in serum homocysteine, a functional marker of impaired folate homeostasis, especially in the setting of decreased dietary folate intake [20, 24]. Because replete folate stores counteract the effects of antifolates, patients homozygous (~10% of some populations) or heterozygous (~40%) for these

polymorphisms are therefore at increased risk for MTX-induced toxicity [7, 10, 25]. Functional polymorphisms have been described in other proteins involved in folate metabolism including the reduced folate carrier, folylpolyglutamate synthetase, dihydrofolate reductase, ATIC, GGH, MS, and MTRR. The possibility that these polymorphisms relate to the toxicity and efficacy of MTX has been the subject of recent reviews [7, 44, 45]. A larger study than the one reported here will be necessary to define the relationship between these SNPs and AMT-related toxicity. Similarly, this study did not have statistical power to detect the effect of the SNPs analyzed on disease outcome.

Conclusions

AMT offers several potential advantages over MTX. AMT is more avidly accumulated by patients' leukemic blasts in vitro than MTX [5, 53], is more completely converted to polyglutamate forms [5, 53], due to a greater affinity for folylpolyglutamate synthetase [14, 34], and is more reliably bioavailable than MTX [5, 23, 40]. The administration schedule of oral AMT (two doses 12 h apart) allows facile intensification of the antifolate component of therapy for patients with ALL, and may also make AMT particularly useful in parts of the world where hospitalizations for IV therapy are impractical. Using AMT now in multiagent therapy for the first time, we find that it can be safely incorporated at its MTD, without causing excessive or unpredictable toxicity. Given the potential advantages of AMT, and the acknowledged value of repeated antifolate dosing in therapy for patients with ALL, these data support a larger trial, comparing the efficacy and toxicity of AMT and MTX in multiagent therapy for patients with ALL.

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