

Phase II Trial of Oral Aminopterin for Adults and Children with Refractory Acute Leukemia

Peter D. Cole,^{1,2} Richard A. Drachtman,^{1,2} Angela K. Smith,¹ Sarah Cate,² Richard A. Larson,³ Douglas S. Hawkins,⁴ John Holcenberg,⁴ Kara Kelly,⁵ and Barton A. Kamen^{1,2}

Abstract Purpose: To determine the antileukemic activity of weekly oral aminopterin in patients with refractory acute leukemia; to describe the pharmacodynamic properties of aminopterin; and to contrast the intracellular metabolism of aminopterin and methotrexate by patients' blasts *in vitro*.

Experimental Design: Forty-six patients were enrolled in three strata: children with acute lymphoblastic leukemia (ALL), adults with ALL, and patients with acute myeloid leukemia (AML). Aminopterin was given weekly, in two doses of 2 mg/m², 12 hours apart. Limited sampling pharmacokinetic analysis was done during the first week of therapy. Accumulation of [³H]aminopterin and [³H]methotrexate by leukemic blasts was studied *in vitro*.

Results: Six of 22 children with ALL (27%; 95% confidence interval, 8-47%) had clinically significant responses. None of those with AML and only two of 11 adults with ALL had responses meeting protocol definitions, although peripheral blast counts tended to decrease with therapy in all groups. Mucosal toxicity was minimal, even with limited use of leucovorin rescue. Complete bioavailability of aminopterin was confirmed, with a mean area under the curve of 0.52 ± 0.03 μmol hour/L after oral dosing. No relationship between aminopterin pharmacokinetics and response was seen. *In vitro*, aminopterin showed more consistent metabolism by leukemic blasts to polyglutamates than methotrexate. Lineage-specific differences in the pattern of intracellular antifolylpolyglutamates were observed.

Conclusions: Weekly oral aminopterin has significant activity among children with refractory ALL. With greater cellular accumulation and metabolism, more reliable bioavailability than methotrexate, and tolerable toxicity at this dose and schedule, aminopterin deserves further study as a potent alternative to methotrexate.

Aminopterin (4-amino-4-deoxy-pteroylglutamic acid; NSC 739) was the first drug reported to induce remissions in children with acute leukemia (1). Although aminopterin is a more potent antifolate than methotrexate (4-amino-4-deoxy-10-*N*-methyl-pteroylglutamic acid; NSC 740; refs. 2-8), it was supplanted by methotrexate in the early 1950s because its

toxicity was felt to be unpredictable (4, 6, 7, 9-12). Methotrexate remains a core component of all therapeutic regimens used for patients with acute lymphoblastic leukemia (ALL) and is widely used to treat many other malignant and nonmalignant disorders (13). However, aminopterin may offer advantages over methotrexate, with greater accumulation and metabolism by leukemic blasts *in vitro* (14) and more complete oral bioavailability (15) than has been reported for methotrexate (16, 17).

Because the most accepted role for an antifolate is not as a single agent, but in the setting of multiagent, post-remission therapy, our goal in the clinical development of aminopterin has been predicated on finding a dose and schedule to mimic the divided-dose oral methotrexate that has been used in post-induction therapy for patients with ALL (18, 19). The maximum tolerated dose of aminopterin on our phase I trial was 2 mg/m²/dose q12 hours for two doses weekly (15). Mucosal toxicity was dose limiting at higher doses and ameliorated by dose reduction and/or the addition of leucovorin rescue. The observed toxicity at the maximum tolerated dose was similar to that observed in patients given methotrexate (25 mg/m²) q6h for four doses weekly as a component of therapy for ALL (18).

This phase II trial was conducted to confirm the bioavailability of oral aminopterin and to determine the antileukemic activity of aminopterin in adult and pediatric patients with

Authors' Affiliations: ¹Department of Pediatric Oncology, The Cancer Institute of New Jersey; and ²Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, New Brunswick, New Jersey; ³The University of Chicago, Chicago, Illinois; ⁴Children's Hospital and Regional Medical Center, Seattle, Washington; and ⁵Columbia University, New York, New York
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Note: P.D. Cole is a Damon Runyon-Lilly Clinical Investigator.

B.A. Kamen is an American Cancer Society Clinical Research Professor.

Requests for reprints: Peter D. Cole, Department of Pediatric Hematology/Oncology, The Cancer Institute of New Jersey, 195 Little Albany Street, New Brunswick, NJ 08901. Phone: 732-235-8076; Fax: 732-235-6462; E-mail: colepd@umdnj.edu.

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acute leukemia. In addition, we continue to contrast the accumulation and metabolism of [³H]aminopterin versus [³H]methotrexate by patients' lymphoblasts *in vitro*, with the goal of better understanding the observed differences in clinical potency between the two antifolates.

Materials and Methods

Clinical trial. This was an open-label, single-agent, noncomparative trial to determine if aminopterin is active in the treatment of patients with refractory acute leukemia. Patients were stratified in three treatment groups: stratum 1, children ages <20 years with ALL; stratum 2, adults ages ≥20 years with ALL; and stratum 3, patients of any age with acute myeloid leukemia (AML). Sample size in each stratum was determined using the Gehan two-stage sampling design.

Patients with histologically documented acute leukemia of any subtype, refractory to known effective therapy were eligible. Patients were required to have recovered from all prior acute drug-related toxicity, to have adequate nutritional status (weight above 3rd percentile for age and total serum protein within the reference range), to have adequate hepatic and renal function (total bilirubin <1.5 mg/dL, alanine aminotransferase less than five times the upper limit of normal, serum creatinine normal for age), to have performance status of >50% as measured by the Karnofsky Performance Scale, and to have a predicted life expectancy of >6 weeks. Patients who had previously undergone bone marrow transplantation were required to be at least 3 months from the procedure before enrollment. Patients with severe uncontrolled infections, patients with evidence of significant third-space effusions, patients who have received high-dose methotrexate (≥1g/m²/dose) of >12 times, and pregnant or lactating women were excluded. All patients (or their guardians, in the case of minors) gave signed informed consent, indicating that they were aware of the investigational nature of this study.

Between July 1997 and December 2004, 46 patients were enrolled and treated at five institutions: The Cancer Institute of New Jersey/Robert Wood Johnson University Hospital (New Brunswick, NJ), Children's Hospital and Regional Medical Center (Seattle, WA), The University of Texas Southwestern Medical Center (Dallas, TX), Columbia University (New York, NY), and the University of Chicago (Chicago, IL). The protocol and informed consent were approved by the institutional review board of each institution.

Materials. Intravenous aminopterin and the liquid for oral administration were purified and packaged by Ilex Oncology, Inc. (San Antonio, TX), as previously described (15). During the tenure of the phase II trial, the liquid preparation, which was being analyzed every 4 to 8 weeks for purity, was found to be accumulating folic acid. When the folic acid reached ~5%, we discontinued its use. Knowing that we were reaching the limits of expected shelf life for this i.v. preparation, we had already prepared a 1 mg scored tablet. Aminopterin tablets were supplied by the Cancer Institute of New Jersey. Synthesis of aminopterin tablets was supported in part by an Food and Drug Administration Orphan Products Development grant FD-R-001832-03. Aminopterin was synthesized per the previously reported method of Piper and Montgomery (20), and the powder was pressed into a 1 mg scored tablet by the University of Iowa under IND #49,927. Purity of the aminopterin tablet was assayed at Cancer Institute of New Jersey 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 aminopterin tablets to be >98% pure aminopterin. Folic acid accounts for <0.3% of the total, with breakdown products, such as pterins and PABA, accounting for the remainder.

Treatment. Aminopterin was given weekly at a dose of 2 mg/m²/dose for two doses, 12 hours apart. Patients were instructed to take only clear liquids for a minimum of 30 minutes before and following the administration of oral aminopterin. A minimum of 4 weeks of therapy

was planned for each patient. To avoid antagonizing the antifolate effects of aminopterin, patients were instructed to avoid multivitamins containing folic acid. However, the standard recommended daily allowance of vitamin A was suggested for all patients, because of animal data suggesting that it might selectively protect against the mucosal toxicity of antifolates (21).

The pharmacokinetics of i.v. and oral liquid aminopterin administration were compared during the first 2 weeks of therapy, for the first 24 patients on study. After complete bioavailability was confirmed, the protocol was amended, and all subsequent patients were given oral aminopterin only. The last 16 patients, enrolled after April 2001, received aminopterin tablets rather than aminopterin liquid. Limited-sampling pharmacokinetics of the tablet were done to confirm equality with the oral liquid.

The first 18 patients were given two doses of leucovorin (5 mg/m²/dose) initiated 24 hours after the second aminopterin dose each week. The protocol was then amended, as a result of the Food and Drug Administration-mandated folate supplementation of foods, which caused a significant increase in average plasma (22) and whole blood folate (23). Subsequent patients were given leucovorin rescue only in the event of grade ≥2 mucosal or other nonhematologic toxicity.

Toxicity criteria and reporting. All patients who received at least one dose of aminopterin were assessable for toxicity. Patients were assessed for adverse events at each encounter and instructed to report any suspected adverse event to the investigator. All adverse events occurring after any administration of aminopterin were reported to the study coordinator and followed to the end of the study or until resolution. Toxicity was graded on a scale of 0 to 5, using the National Cancer Institute Common Toxicity Criteria (CTC), version 2.0.

Response criteria. All patients were evaluated for response. At the responsible investigators' discretion, some patients with obvious clinical progression during the initial 4 weeks of aminopterin were removed from the study and counted with those patients with progressive disease. In the absence of clinically evident disease progression, bone marrow aspiration was done after 4 weeks of therapy. Bone marrow biopsies were required to assess cellularity when the aspirate was hypocellular.

Patients with AML and ALL were evaluated using different response criteria. In patients with ALL, responses were defined using evaluation of bone marrow, peripheral blood counts, physical examination, and performance status. Complete response was defined as a reduction to ≤5% blasts in the bone marrow, with normal blood counts (hemoglobin ≥ 11 g/dL, absolute neutrophil count > 1,500/μL, platelets ≥ 100,000/μL, and no peripheral blasts), absence of organomegaly, absence of leukemia-related symptoms, and Karnofsky performance status of >50%. Partial response was defined as reduction to <25% blasts in the bone marrow, with hemoglobin ≥ 7 g/dL, absolute neutrophil count > 500/μL, platelets ≥ 25,000/μL, ≤5% peripheral blasts, and absence of organomegaly beyond the umbilicus. Clinical improvement was defined by improvement of either the bone marrow to <25% blasts or of the blood counts from initially abnormal values to those necessary for definition of a partial response (hemoglobin ≥ 7 g/dL, absolute neutrophil count > 500/μL, platelets ≥ 25,000/μL, and ≤5% peripheral blasts).

For patients with AML, complete response was defined by an absence of clinically evident extramedullary disease and bone marrow with adequate cellularity (>20%), with ≤5% blasts and no Auer rods. Partial response was defined by a cellular bone marrow with either the presence of Auer rods or between 5% and 25% blasts. No peripheral blood criteria were included in the definition of response among patients with AML.

Pharmacokinetics. To confirm oral bioavailability of aminopterin, limited sampling pharmacokinetic analysis was conducted, as previously described (15). Briefly, 2 mL of blood was collected in an EDTA tube before and 30, 60, 180, and 300 minutes after the aminopterin was administered. For the first 24 patients, the kinetics of oral and i.v. aminopterin were compared within the first 2 weeks of therapy. For the

Table 1. Characteristics of patients enrolled

	Stratum		
	ALL, <20 y	ALL, ≥20 y	AML
<i>n</i>	22	11	13
Median age (range)	9.7 (1-20)	35.4 (21-61)	46.9 (2.6-73)
Male (%)	50	50	39
T phenotype (%)	12 (55)	1 (10)	NA
Median duration of aminopterin therapy, wk (range)	4 (1-19)	6 (4-8)	3 (1-8)

NOTE: Patients were enrolled in three strata: children with ALL, adults with ALL, and all those with AML.
Abbreviation: NA, not applicable.

subsequent 22 patients, who received oral aminopterin only, kinetics were measured after a single oral dose.

Aminopterin concentrations in plasma were determined in the Pediatric Oncology Laboratory at the Cancer Institute of New Jersey, using a radioligand binding assay, as previously published (24). Although the assay was initially developed to measure methotrexate, aminopterin and methotrexate are equivalent in this competitive binding assay, with an absolute limit of detection of 0.05 to 0.1 pmol, depending on the specific activity of the tritiated methotrexate. Noncompartmental analysis was done to calculate the area under the curve (AUC), using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA; <http://www.graphpad.com>).

Accumulation of aminopterin polyglutamates and methotrexate polyglutamates in vitro. Accumulation of [³H]aminopterin and [³H]methotrexate by blasts isolated from patients before treatment with aminopterin was measured as previously described after 24 hours of incubation *in vitro* (14). Briefly, lymphoblasts were isolated from bone

marrow or blood by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density-gradient centrifugation, washed with HBSS, counted on a Coulter counter, and incubated at a concentration of $5 \times 10^6/2$ mL media containing the radiolabeled antifolate of interest at 37°C in a humidified atmosphere with 5% CO₂. After 24 hours, the cells were then collected, washed thrice in 5 mL HBSS, and suspended in 2 mL HBSS. An aliquot was counted in a hemocytometer and subjected to viability determination by trypan blue exclusion. Intracellular content of [³H]methotrexate or [³H]aminopterin was determined by scintillation counting and calculated as pmol/10⁶ cells. Aminopterin and methotrexate polyglutamates were then separated by high-performance liquid chromatography, using a C18 column by reverse-phase chromatography as previously detailed (25).

Due to temporary unavailability of labeled aminopterin and/or insufficient numbers of viable blasts for analysis, accumulation of [³H]aminopterin and [³H]methotrexate was assayed for only 12 patients on the phase II aminopterin trial. Additional analysis of intracellular metabolism of methotrexate and aminopterin to polyglutamates has also been done using blasts isolated from different patients (*n* = 80) with acute leukemia at the time of initial diagnosis, after institutional review board–approved informed consent was given to study their blasts. The data reported below includes the results of analysis of both these newly diagnosed patients (not previously presented) and those enrolled on the phase II trial of aminopterin.

Results

Patients. Between July 1997 and October 2004, >400 doses of aminopterin were administered to 46 patients enrolled on this phase II trial. Patient characteristics are summarized in Table 1; more detailed information regarding the eight patients who responded to aminopterin is presented in Table 2. Twenty-seven patients (59%) were White, 10 (22%) were Hispanic, seven (15%) were African American, and the remaining two were Asian and American Indian, respectively. Twenty-five patients (54%) completed at least

Table 2. Clinical characteristics of patients who responded to aminopterin therapy

Patient ID	Age (y)	Sex	Race	ALL phenotype	Initial treatment regimen	Condition on study entry	CBC on study entry				
							WBC ($\times 10^3/\mu\text{L}$)	Blasts (cells/ μL)	ANC (cells/ μL)	Hg (g/dL)	Plts ($\times 10^3/\mu\text{L}$)
1001	11	M	AA	T	POG 9086	First relapse; multiagent reinduction failure	1	290	310	9.8	39
6004	12	M	W	T	CCG 1961	First relapse; multiagent reinduction failure	2.8	360	1,290	11.7	232
1007	9	F	AA	T	POG 9404	Induction failure; failed multiple salvage attempts	1.8	0	860	9.5	191
6012	14	M	AA	pre-B	CCG 1962	Third relapse (first marrow relapse)	1.7	0	220	9.3	32
3002	8	F	W	pre-B	CCG 1922	Fifth relapse (first marrow relapse)	1.7	1,000	50	11.5	138
1002	19	M	W	T	POG 9086	First relapse; multiagent reinduction failure	2.9	1,100	550	10.3	50
6015	20	M	H	pre-B	DFCI 91-01	First relapse; multiagent reinduction failure	22.4	14,000	220	13.5	58
2001	61	M	W	pre-B	CALGB 8811	First relapse; multiagent reinduction failure	1.8	200	270	8.9	21

NOTE: Patients were enrolled in three strata: children with ALL, adults with ALL, and all those with AML.
Abbreviations: AA, African American; W, White; H, Hispanic; Hg, hemoglobin; Plts, platelets.

Table 3. Responses to aminopterin by treatment strata

	Stratum		
	ALL, <20 y	ALL, ≥20 y	AML
Progression/no response	17	9	13
Clinical improvement	2	2	0
Partial response	2	0	0
Complete response	2	0	0

NOTE: Patients were evaluated every 4 weeks while on study. Response to aminopterin was defined by changes in peripheral blood counts, physical examination, and bone marrow examination.

one full cycle (2 doses/wk × 4 weeks). With the exception of one adult with AML who died of systemic infection, those patients who did not complete a full cycle stopped due to disease progression and not toxicity.

One patient was given concurrent hydroxyurea for hyperleukocytosis, in violation of the protocol prohibition against concurrent systemic chemotherapy. This patient received six doses of aminopterin over 3 weeks before being taken off therapy for clinically evident progressive disease. This patient developed grade 3 mucositis. Another patient, a child with pre-B lineage ALL, enrolled twice. She had a clinical improvement during 12 weeks of aminopterin therapy. One year later, she enrolled again but had no response despite 8 weeks of therapy.

Mucosal toxicity. The majority of patients ($n = 27$, 59%) experienced no mucositis. Six (13%) developed grade 1 mucositis, and 10 (22%) developed grade 2 mucositis. Grade 3 ($n = 1$) or grade 4 ($n = 2$) mucositis was seen infrequently (6%). Twenty-eight patients enrolled after the protocol amendment requiring the addition of leucovorin rescue only for the treatment of grade 2 mucositis. Of these, 16 (57%) required no leucovorin during the first 4 weeks of aminopterin therapy.

Other toxicity. Adverse events while on study were largely due to marrow failure, either secondary to leukemia or the myelosuppressive effect of aminopterin. Nonhematologic

toxicities directly related to aminopterin were few in number. One patient with AML developed tumor lysis syndrome during the first week of therapy and died of infectious complications (CTC grade 5 neutropenic sepsis). Three patients developed skin rashes: one with CTC grade 2 erythematous macular rash covering <50% of the body surface, one with varicella zoster (CTC grade 3 infection), and another who suffered a sunburn (CTC grade 2) during a trip to Honolulu. One patient with AML had a deep vein thrombosis (CTC grade 3) that developed in the context of clinical sepsis. Three patients developed asymptomatic grade 2 to 3 hepatotoxicity (serum bilirubin >1.5 and <10 times the upper limit of normal and/or serum aspartate aminotransferase/alanine aminotransferase >2.5 and <20 times the upper limit of normal).

Responses. The highest response rate (27%; 95% confidence interval, 8.5-46%) was seen among children with ALL (Table 3). The time to best response among these children ranged from 8 to 12 weeks. Hematologic variables among those meeting protocol response criteria are shown in Table 4. One of the two children classified as having a partial response (a 14-year-old African American male with multiply relapsed, pre-B lineage ALL) had a bone marrow after his second 4-week cycle that straddled the criteria dividing M_1 and M_2 , with 4% lymphoblasts by morphology and 6% by flow cytometry. With normal peripheral counts and no peripheral blasts, he therefore, might have been considered a complete response if we used the blast counts determined by histology. The response criteria in the protocol, however, did not specify which method should be used to determine blast percentage in the marrow. His bone marrow after the 12th week showed stable disease, with 7% lymphoblasts by flow cytometry. This patient remained on study for a total of 14 weeks, with minimal toxicity before progressing. He required no leucovorin for mucositis until after his 13th week of aminopterin.

Only two adults with ALL, ages 20 and 61 years, both with B-lineage ALL, had clinical improvement during aminopterin therapy, with normalization of peripheral blood counts (Table 4) and the physical examination but no bone marrow examination with <25% blasts. One additional adult with Sézary syndrome and circulating blasts had symptomatic

Table 4. Hematologic responses to aminopterin meeting protocol criteria

Patient ID	Best response	Time on treatment (wk)	CBC at time of best response					BM blasts (%)	Reason off study
			WBC ($\times 10^3/\mu\text{L}$)	Blasts (cells/ μL)	ANC (cells/ μL)	Hg (g/dL)	Plts ($\times 10^3/\mu\text{L}$)		
1001	CR	19	3.3	0	1,550	11.4	232	2	BMT
6004	CR	12	4.5	0	3,290	13.2	233	4	BMT
1007	PR	12	3.4	400	1,770	12.8	307	4	PD
6012	PR	14	3.2	0	1,570	12.5	144	6*	PD
3002	Impr	12	2.9	0	2,349	10.9	185	20	PD
1002	Impr	13	1.9	0	950	10.2	101	43	BMT
6015	Impr	7	1.2	40	520	9.8	42	48	PD
2001	Impr	8	1.3	0	680	10.6	96	40	PD

NOTE: Complete blood counts and bone marrow blast percentage at time of best response, along with duration of aminopterin therapy and reason for coming off study. Abbreviations: BMT, elective withdrawal from study to undergo allogeneic bone marrow transplantation; PD, progressive disease; CR, complete response; PR, partial response; Impr, improvement; BM, bone marrow; Hg, hemoglobin; Plts, platelets; ANC, absolute neutrophil count.

*Patient 6012 had 4% blasts by histology and 6% bone marrow cells showing the diagnostic aberrant phenotype by flow cytometry, as described in the text.

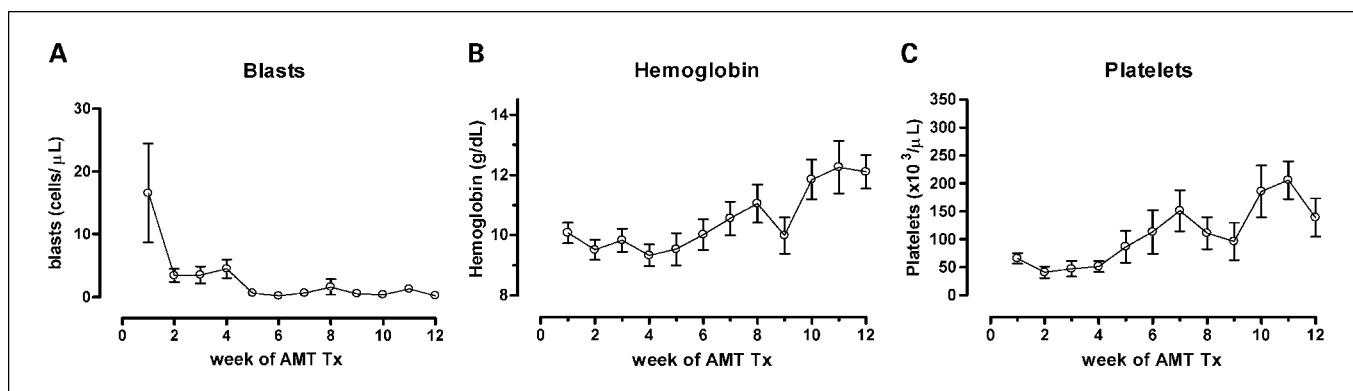


Fig. 1. Hematologic responses to aminopterin therapy. Blood counts were measured at least weekly during therapy with aminopterin. Points, mean; bars, SE. Peripheral blast counts (A) tended to decrease during therapy with aminopterin. In contrast, hemoglobin (B) and platelets (C) tended to increase.

improvement and remained on therapy for 2 months before electing to enroll in another therapeutic protocol.

Combining the children and adults with ALL, the response rate among patients with T-lineage ALL (two complete response, one partial response, and one improved in 13 patients) was greater than that among those with B-lineage disease (one partial response and three improved in 20 patients). This difference is not statistically significant ($P > 0.5$, Fisher's exact test), but this trial was not powered to detect any difference in response among patients with different leukemic subtypes.

There were no responses among 13 patients with AML. As noted above, one patient with AML developed tumor lysis syndrome and pancytopenia after his first dose, suggesting rapid reduction in tumor burden. He died of infectious complications before he could be evaluated for response. Another was on therapy with stable disease for 2.5 months before progressing.

Among patients in each strata, peripheral blast counts tended to decrease during therapy with aminopterin, whereas hemoglobin and platelet counts tended to increase toward normal values (Fig. 1).

Pharmacokinetics. A comparison of the pharmacokinetics after i.v. and oral administration was done in the first 24 patients (Fig. 2). In patients with matched i.v. and oral pharmacokinetics, no significant difference in AUC was observed, with mean oral bioavailability of 96.8%. Among all patients, the mean AUC (\pm SE) after oral administration was 0.52 ± 0.04 μ mol hour/L, which is not statistically different from that seen after administration of i.v. aminopterin at the same dose (2 mg/m^2) on the phase I trial (15). Pharmacokinetic analysis revealed no significant difference between the tablet ($n = 16$) and liquid formulations ($n = 29$) in AUC.

No significant difference was seen between those patients with clinical responses to aminopterin and those who did not respond, in mean peak serum aminopterin concentration (0.166 ± 0.018 versus 0.167 ± 0.035 μ mol/L) or AUC (0.48 ± 0.04 versus 0.40 ± 0.06 μ mol hour/L).

Accumulation of aminopterin and methotrexate polyglutamates in vitro. Earlier reports have related clinical outcome with methotrexate accumulation by leukemia blast cells, either incubated with $1 \mu\text{mol/L}$ *in vitro* or after an infusion of methotrexate before the start of standard therapy (i.e., an *in situ* incubation; refs. 26, 27). We therefore assessed

$[^3\text{H}]$ methotrexate and $[^3\text{H}]$ aminopterin accumulation and metabolism in blast cells whenever sufficient cell numbers were available before therapy. Those patients with clinical responses to aminopterin tended to show greater accumulation of $[^3\text{H}]$ aminopterin by their leukemic blasts *in vitro* (mean \pm SE: 0.79 ± 0.19 pmol/ 10^6 cells; $n = 6$) than those who did not respond (mean: 0.41 ± 0.09 pmol/ 10^6 cells; $n = 6$; $P = 0.09$, two-tailed *t* test).

To contrast accumulation and metabolism by blasts of different leukemic types, blasts from patients on this phase II trial ($n = 12$) as well as others with newly diagnosed leukemia ($n = 80$) have been tested and the data combined. After 24 hours of incubation with either $1 \mu\text{mol/L}$ $[^3\text{H}]$ methotrexate or $0.1 \mu\text{mol/L}$ $[^3\text{H}]$ aminopterin, there was no significant difference in total accumulation between blasts from patients with pre-B phenotype ALL, T-lineage ALL, and AML (Table 5). As noted by others (28–30), we find differences in the degree to which each leukemia subtype converts methotrexate to polyglutamates. Lymphoblasts from patients with pre-B ALL are better able to metabolize methotrexate to methotrexate polyglutamates than T lymphoblasts, and both groups of patients' lymphoblasts metabolized more methotrexate than myeloblasts (Table 5). In contrast, aminopterin was metabolized consistently to polyglutamates by both lymphoblast subsets (median polyglutamate fractions of 100%), and the drop-off in

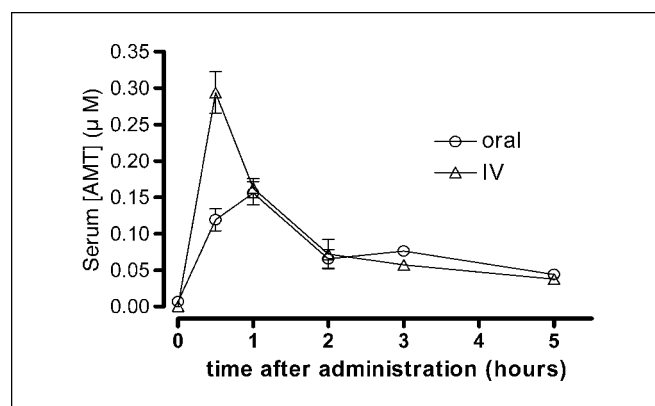


Fig. 2. Aminopterin pharmacokinetics. Mean serum aminopterin concentration after an oral ($n = 41$, \circ) or i.v. ($n = 28$, \triangle) dose of 2 mg/m^2 . Mean AUC \pm SE was 0.52 ± 0.03 and 0.51 ± 0.03 μ mol hour/L after oral and i.v. dosing, respectively. Points, mean; bars, SE.

Table 5. Accumulation and metabolism of aminopterin and methotrexate by patients' leukemic blasts

Antifolypolyglutamate (extracellular concentration)	<i>n</i>	Total intracellular antifolypolyglutamate (pmol/10 ⁶ cells), median (range)	% Found as antifolypolyglutamate			
			Median (range)	Compared with Pre-B ALL (<i>P</i>)	Aminopterin compared with methotrexate (<i>P</i>)	
Methotrexate (1 μmol/L)	Pre-B ALL	52	0.64 (0.06-3.55)	96 (28-100)	—	
	T-ALL	21	0.65 (0.09-2.01)	89 (6-100)	<0.05	
	AML	19	0.45 (0.16-3.56)	69 (44-100)	<0.01	
Aminopterin (0.1 μmol/L)	Pre-B ALL	24	0.33 (0.08-1.90)	100 (83-100)	—	<0.01
	T-ALL	13	0.38 (0.11-1.17)	100 (73-100)	NS	<0.05
	AML	12	0.35 (0.14-0.51)	97 (36-100)	<0.05	<0.01

NOTE: Bone marrow leukemic blasts isolated from patients were incubated with 1 μmol/L [³H]methotrexate or 0.1 μmol/L [³H]aminopterin *in vitro*. After 24 hours, the cells were collected, washed, and lysed. An aliquot was counted scintillation counter to determine total accumulation, expressed as pmol/10⁶ viable cells. Antifolypolyglutamates were separated from the parent compounds by high-performance liquid chromatography and compared with known, unlabeled standards.

Abbreviations: B-ALL, B-lineage ALL; T-ALL, T-phenotype ALL; NS, not significant.

the myeloblasts was smaller than that seen for methotrexate. Within each subset, the percentage of aminopterin found as a polyglutamate was significantly greater than the corresponding methotrexate polyglutamate fraction ($P < 0.05$ for each comparison, two-tailed Mann-Whitney U test).

After a high-performance liquid chromatography separation, interesting differences between the distribution of intracellular aminopterin and methotrexate polyglutamates were also noted. In lymphoblasts from patients with B-lineage ALL, methotrexate polyglutamates from glu₂ through glu₅ are found in increasing frequency with increasing chain length, whereas the corresponding aminopterin polyglutamates are found in decreasing frequency (Fig. 3A). However, for both aminopterin and methotrexate, long-chain polyglutamates (≥ 3 glutamate residues) exceed 60% of the total intracellular antifolate pool.

The pattern of aminopterin polyglutamates in pre-B ALL blasts differs dramatically from that seen in T-phenotype ALL or AML (Fig. 3B and C), where aminopterin-glu₂ predominates and little longer-chain aminopterin is seen (<30% of the total). It is striking that under similar conditions, the natural ligand methyl tetrahydrofolate is metabolized completely to very long-chain polyglutamates, predominantly -glu₅₋₇ (data not shown).

Discussion

Aminopterin was replaced by methotrexate in the 1950s, because the latter had a better therapeutic index in murine models (31), and the clinical toxicity of aminopterin was less predictable (4, 6, 7, 9–12). No randomized clinical trial was done to compare the two antifolates. In retrospect, aminopterin may have been discarded prematurely. The erratic clinical toxicity of aminopterin five decades ago may have been the result of variable contamination with folate (32, 33), providing unpredictable degrees of rescue. This problem has been eliminated in more modern preparations. We confirm the purity of the aminopterin tablets at least quarterly using three methods: UV spectrum, radioligand binding assay using dihydrofolate reductase and radiolabeled methotrexate, and high-performance liquid chromatography. When we found significant decomposition of the initial liquid preparation over 3 years from initial

formulation, the impurity seemed to be folic acid.⁶ In 4 years, this decomposition has not yet been observed with the tablet.

In the early era of antifolate chemotherapy (1948-1951), as many as 66% of newly diagnosed patients responded to daily oral or i.m. aminopterin (1). Clinical resistance to antifolates upon relapse was also described (4, 5, 10, 11), and much of the biochemical basis for antifolate resistance has been elucidated over the subsequent decades (34, 35). We initially brought aminopterin back to the clinic, because laboratory data suggest that it may offer advantages over methotrexate for some patients with ALL. At identical extracellular concentrations, more aminopterin than methotrexate is accumulated by blasts from patients with pre-B ALL, T-lineage ALL, and AML (14). In addition, less interpatient variability is observed in the degree of metabolism of aminopterin to aminopterin polyglutamates than of methotrexate to methotrexate polyglutamates. It is likely that both of these observations are explained by differences in affinity for folypolyglutamate synthase, the enzyme that catalyzes the sequential addition of glutamate residues to folate and classic antifolates, effectively trapping them within the cell. Aminopterin has 20-fold greater affinity for folypolyglutamate synthase than methotrexate and a higher ratio of V_{max}/K_m (36–38).

Excellent oral bioavailability of aminopterin was observed in the phase I trial (15), a finding confirmed in this phase II trial. In addition, we note that the interpatient variability in oral pharmacokinetics (coefficient of variation = 34.7%; $n = 26$) is less than that which has been observed for methotrexate (up to 59%), at approximately equipotent doses (16, 17, 39).

We now describe for the first time the distribution of aminopterin polyglutamates after metabolism by patients' leukemic blasts. Consistent with earlier data with murine cells (40, 41), diglutamate predominance was observed in blasts from patients with T-phenotype ALL or AML. However, we note that in blasts from patients with B-lineage ALL, longer chain polyglutamates (aminopterin-glu₃₋₅) were seen with a frequency similar to that of the diglutamate. The reasons for this lineage-specific difference in aminopterin polyglutamylation are not clear. With methotrexate as a substrate, quantitative

⁶ Unpublished data.

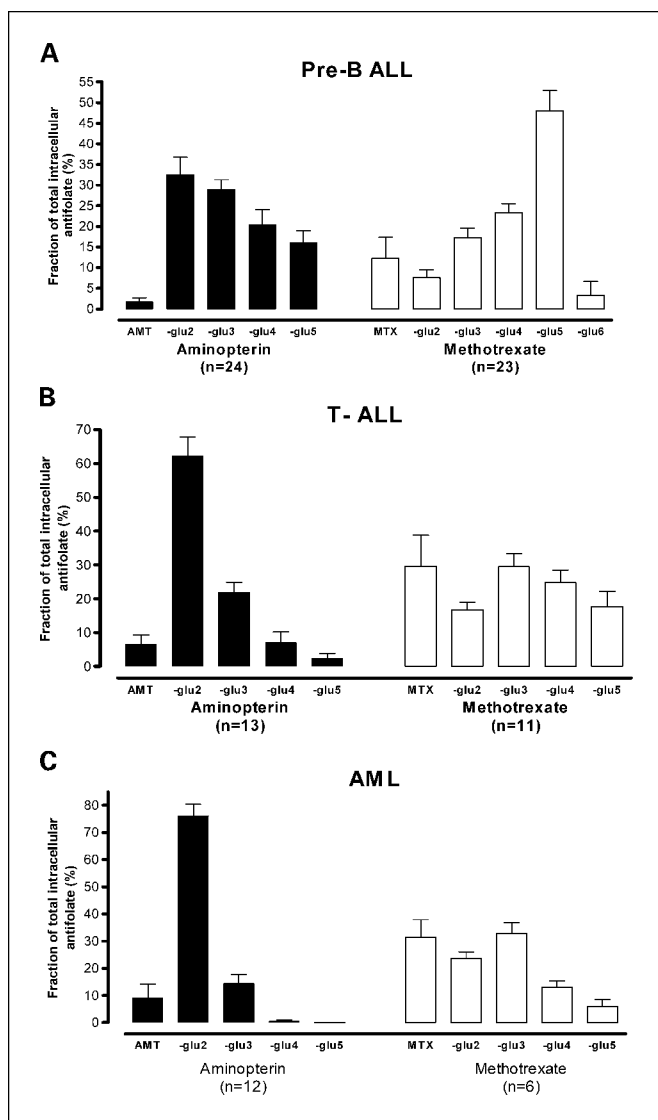


Fig. 3. Intracellular antifolypolyglutamates formed *in vitro* by patients' leukemic blasts. Bone marrow leukemic blasts isolated from patients at initial diagnosis were incubated with 1 $\mu\text{mol/L}$ [^3H]methotrexate or 0.1 $\mu\text{mol/L}$ [^3H]aminopterin *in vitro*. After 24 hours, the cells were collected, washed, and lysed. Antifolypolyglutamates were separated by high-performance liquid chromatography and compared with known, unlabeled standards. For each polyglutamate species, the mean fraction of the total intracellular antifolate is indicated. Columns, mean; bars, SE. *A*, accumulation by leukemic blasts from patients with B-lineage ALL. *B*, accumulation by leukemic blasts from patients with T-lineage ALL. *C*, accumulation by leukemic blasts from patients with AML.

differences in polyglutamylation between B-lineage ALL and T-phenotype ALL or AML have been related to quantitative differences in folypolyglutamate synthase expression (29, 42, 43) and to qualitative differences in folypolyglutamate synthase affinity for methotrexate (44).

The differences we observed between aminopterin and methotrexate polyglutamylation profiles in leukemic blasts raise a paradox. It is generally accepted that longer chain folypolyglutamates or antifolypolyglutamates are preferentially retained within the cell longer and are more toxic (45–47). However, at equimolar extracellular concentrations, steady-state aminopterin accumulation is greater than that of methotrexate (14), despite a shorter average polyglutamate chain length.

It is possible that there is a disparity in the affinities of aminopterin and methotrexate for efflux mechanisms. It is notable that although aminopterin polyglutamates had shorter average chain length than methotrexate polyglutamates in blasts of each leukemic subtype, conversion of the parent compound to at least the diglutamate was more consistent for aminopterin than methotrexate. Metabolism to even the diglutamate form is known to significantly retard cellular efflux of classic antifolates like methotrexate and aminopterin (40). Methotrexate but not methotrexate diglutamate is a substrate for efflux pumps, such as the human multidrug resistance proteins MRP3 (48) and MRP4 (49). In addition to affecting efflux rates, the different pattern of intracellular polyglutamate profiles might cause differences between the two antifolates in efficiency of inhibition of target enzymes or subcellular localization. Ongoing studies in our laboratory are addressing these questions.

With greater cellular accumulation and metabolism, more reliable bioavailability than methotrexate, and tolerable toxicity at this dose and schedule, aminopterin deserves further study as a potent alternative to methotrexate. Our current results show aminopterin retains significant activity among children with refractory ALL. Greater response rates, among untreated patients, were seen historically (1, 3, 5, 6) when aminopterin was given on a daily schedule at lower doses (0.5–1 mg i.m. daily \times 5 for up to 3 weeks), not much greater than the RDA for folic acid. As noted above, the dose and schedule used on this trial (4 mg/m²/wk in two divided doses, 12 hours apart) were developed explicitly for the purpose of inclusion in post-induction multiagent therapy. It is possible that patients with refractory ALL would benefit from a trial reexploring the earlier aminopterin regimen. For now, based on these data, we are testing whether aminopterin can be safely substituted for systemic methotrexate after induction in multiagent therapy for children with newly diagnosed ALL at high risk of relapse (50).

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