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ORIGINAL ARTICLE

Clinical potency of methotrexate, aminopterin, talotrexin and pemetrexed in childhood leukemias

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Abstract
Purpose Renewed interest in antifols for the treatment of childhood cancers has resulted from identification of novel antifols with broad spectrums of anti-cancer activity and from re-evaluation of the original clinical antifol, aminopterin. In this pre-clinical study we evaluated the in vitro activity of both traditional antifols (methotrexate, aminopterin) and novel antifols (pemetrexed, talotrexin) in childhood acute leukemias and lymphomas.
Methods We compared the in vitro cytotoxicity of methotrexate, aminopterin, pemetrexed, and talotrexin in a panel of six pediatric leukemia and lymphoma cell lines using the sulforhodamine B assay. In addition to defining a 50% growth inhibitory concentration (IC_{50}) for a 120-h drug exposure, we contrasted the activity of the drugs in the context of clinically achievable (tolerable) drug exposures using the area under the plasma concentration–time curve (AUC). We defined each agent's clinical potency index (CPI) as the AUC achieved with standard pediatric dosing regimens divided by the in vitro IC_{50}.
Results Across all cell lines, talotrexin (median IC_{50} 7 nM) and aminopterin (median IC_{50} 17 nM) had lower IC_{50}'s than methotrexate (median IC_{50} 78 nM) and pemetrexed (median IC_{50} 155 nM). However, the CPI for methotrexate (median 0.9) was significantly greater than that for aminopterin (median 0.4). In contrast, pemetrexed had a significantly better CPI (median 13) than the traditional antifols.
Conclusions Aminopterin does not appear to offer any advantage over methotrexate for the treatment of childhood ALL. Further study of pemetrexed in childhood leukemias is warranted.

Keywords Antifol · Methotrexate · Aminopterin · Pemetrexed · Talotrexin · In vitro

Introduction

Since the introduction of aminopterin (4-amino-4-deoxypteroylglutamic acid; NSC 739) in 1948, antifols have had a long-standing and well established role in the treatment of childhood cancers [10]. By the 1960s, methotrexate (4-amino-4-deoxy-10-*N*-methyl-pteroylglutamic acid), which has a more predictable toxicity profile than aminopterin, had become a cornerstone of the treatment for childhood acute lymphoblastic leukemia (ALL) and for non-Hodgkins lymphoma [4, 17, 23]. A number of other antifols with distinct biochemical properties have since been developed and have undergone initial clinical evaluation in children with cancer [1, 2, 9, 14, 30]. More recently, talotrexin (PT-523, Na-(4-amino-4-deoxypteroyl -Nd-hemiphthaloyl-L-ornithine)), a novel antifol that has characteristics of classical and non-classical antifols (Fig 1), has undergone phase 1 studies in adults with refractory leukemia [11]. Pemetrexed (Alimta, MTA, LY231514, N-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl] benzoyl L-glutamic acid) is a structurally novel antifol that has different target enzyme affinities than MTX, AMT, and TLX. Pemetrexed has been approved for the treatment of

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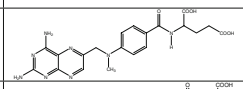
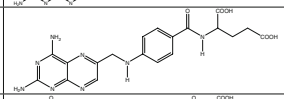
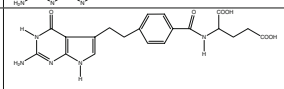
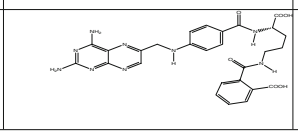
Fig. 1 Structure and chemical properties of methotrexate, aminopterin, pemetrexed, and talotrexin

Antifol	Structure	Transmembrane Transport	Target Enzyme	Polyglutamated	References
MTX		RFC FR system	DHFR, TS, GARFT	Yes	[21, 22]
AMT		RFC FR system	DHFR	Yes	[22, 27]
PXD		RFC FR system	TS, GARFT, DHFR	Yes	[28, 32]
TLX		RFC	DHFR	No	[26]
MTX=Methotrexate, AMT=Aminopterin, PXD=Pemetrexed, TLX=Talotrexin RFC=Reduced Folate Carrier, FR=Folate Receptor, TS=Thymidylate synthase, DHFR=Dihydrofolate reductase, GARFT=Glycinamide ribonucleotide formyltransferase					

non-small cell lung cancer and, in combination with cisplatin, for the treatment of malignant pleural mesothelioma [6, 12]. Pemetrexed has been evaluated by the Children's Oncology Group (COG) Phase 1 Consortium in a phase 1 trial [19].

In addition to investigation of novel antifols in childhood cancer, there has been renewed interest in studying the role of aminopterin for the treatment of childhood ALL. This has been based on the known greater potency of aminopterin compared with methotrexate, coupled with additional mechanistic and early clinical trial data [7, 8, 24].

Despite the important role of antifols for the treatment of childhood hematologic malignancies, a comparison of the relative cytotoxicity of these agents has not been undertaken. We therefore investigated the in vitro profiles of methotrexate, aminopterin, pemetrexed, and talotrexin in a panel of six common pediatric leukemia and lymphoma cell lines. As potency does not equate with efficacy, we analyzed our data in the context of drug exposures known to be clinically tolerable.

Materials and methods

Materials

Six cell lines representing three pediatric tumor types were used: NALM-6 and NALM-16 (pre-B ALL), JURKAT and CEM (T-cell ALL), RAMOS and NAMALWA (Burkitt's lymphoma). CEM, RAMOS, and NAMALWA cell lines were purchased from American Type Tissue Collection (ATCC), Manassas, VA. NALM-6, NALM-16, and JURKAT cell lines were kindly provided by Dr. Stephen Grupp of The Children's Hospital of Philadelphia. The cell lines were grown in RPMI-1640 containing 10% fetal bovine serum, 1% glutamine, 1% HEPES, 1% sodium pyruvate, and 1% nonessential amino acid solution. All cell lines were tested for mycoplasma contamination using MycoAlert® mycoplasma detection assay from Cambrex (East Rutherford, NJ). Methotrexate and aminopterin were purchased from Sigma (St. Louis, MO), pemetrexed (Alimta, LY231514) was kindly provided by Eli Lilly (Indianapolis, IN) and talotrexin (PT-523) was kindly provided by Hana Biosciences (South San Francisco, CA).

Sulforhodamine (SRB) assay

Each cell line was studied in growth inhibition experiments using 96-well microtiter plates. As antifols are schedule dependent, preliminary experiments were aimed at defining the longest duration of exposure that would allow for continuous logarithmic phase growth of cells without changing of the culture media while maintaining a linear relationship between SRB optical density and cell number. Twenty-four hours after cell plating, the cell lines were exposed to the antifol for 120 h (three replicates per experiment). To ensure that a complete sigmoidal survival-concentration curve could be observed, the following drug concentrations were studied: MTX (0.002–5 μM), AMT (0.0001–1 μM), PXD (0.0003–10 μM), TLX (0.0002–0.5 μM). Experiments were repeated at least twice.

Survival-concentration curves were generated using the SRB assay [18, 31]. Following 120-h drug exposure, the microtiter plates were first centrifuged at 800 rpm at 4°C [18]. 50 μL of cold 50% trichloroacetic acid (TCA) (4°C) was then added to the wells at the liquid air interface of each well to produce a final TCA concentration of 10%. The cell culture plates were incubated for 30 min at 4°C and then washed three times with distilled water. Once plates were air dried, 100 μL of 0.4% SRB stain containing 1% acetic acid was added to each well. The plates were then incubated for 30 min at room temperature and

washed with 1% acetic acid. Once the stained plates were air dried, 100 μL of 10 mM Tris Base was added to each well and plates were gently agitated for 5 min. The optical density was then read using a Molecular Devices VERSAmax (Sunnyvale, CA) microplate reader at 520 nm. The background signal from media-alone controls was subtracted and data were normalized to untreated cells. The 50% growth inhibitory concentration (IC_{50}) was determined by fitting a four parameter logistic equation to the data:

$$\%\ \text{survival} = \left[(E_{max} - E_{min})/(1 + (\text{dose}/EC_{50})^{\text{slope}}\right] + E_{min}$$

where E_{max} and E_{min} are the concentrations at which maximum and minimum cytotoxicity are observed, respectively, and EC_{50} is the concentration at which 50% of the maximum cytotoxicity is attained $(E_{min} + E_{max})/2$.

Clinical potency index

To account for known differences in the clinical tolerability of these antifols, the survival-concentration data was analyzed in the context of human drug exposure and tolerability data. We defined *clinical potency index* (CPI) as the area under the plasma concentration–time curve (AUC) achieved with standard pediatric dosing regimens divided by the in vitro IC_{50} ($CPI = AUC_{clinical}/IC_{50}$). $AUC_{clinical}$ represents the cumulative AUC calculated from the highest total dose in a 21-day cycle that has been shown to be safe and tolerable in children. A higher CPI can result from either a lower in vitro IC_{50} or a higher clinically achievable drug exposure (AUC). The dose and schedules utilized for calculation of the CPI is shown in Table 1. Two different dose-schedules of methotrexate were selected: an oral schedule for comparison with oral aminopterin (25 mg/m^2/dose every 6 h for four doses every week, [7]), and an intravenous schedule (100–200–300 mg/m^2 days 1,8 and 15) [20] for comparison with intravenous pemetrexed and talotrexin. The latter intravenous schedule for methotrexate was selected as it represents the highest commonly utilized intravenous methotrexate dose regimen administered without leucovorin rescue.

Results

The cytotoxicity profiles for antifols studied are shown in Fig. 2. Talotrexin was the most potent of the antifols with a median (range) IC_{50} of 7 (5–13) nM, followed by aminopterin 17 (8–57) nM, methotrexate 78 (33–133) nM and pemetrexed 155 (44–284) nM. When the antifols were compared in the context of clinically achievable drug exposures utilized in standard pediatric dosing regimens, pemetrexed and talotrexin had the highest CPI with a median (range) of 13 (6–40) units and 12 (6–14) units, respectively (Fig. 3). Aminopterin had the lowest overall CPI of all antifols studied (Fig. 4).

Discussion

Using the CPI, a method that incorporates existing human clinical pharmacologic data, we studied the in vitro cytotoxicity of a spectrum of antifols in a panel of common pediatric leukemia and lymphoma cell lines. While there are indeed limitations to evaluating cancer drugs in cell lines in vitro, the in vivo analysis of antifols is significantly complicated by the very high circulating concentrations of thymidine and folate in mouse models [5, 15]. Such high circulating thymidine concentrations, approximately ten-fold greater in mice than in humans, are known to limit the ability to effectively study the anti-cancer effect of antifols in murine models [15].

By evaluating the in vitro cytotoxicity data of each drug in the context of clinically achievable drug exposures that encompasses toxicity through determination of a maximum tolerated dose, our approach may be a better predictor for efficacy than analyses based solely on in vitro data. As expected, our results demonstrated that the IC_{50} of AMT was less than MTX in all of the leukemia cell lines studied, supporting prior observations that AMT is a more potent antifol than MTX. However, when these two agents were compared using the CPI, which incorporates the maximum dose of the antifols that has been shown to be tolerable in children (AMT: 2 mg/m^2 every 12 h for two doses; and

Table 1 Clinical schedules of administration with corresponding total dose and AUC data

	Dose schedule (21-day cycle)	Total dose (mg/m^2)	AUC (μM * h)	Reference
$Methotrexate_{po}$	25 mg/m^2 (po) q6h ×4 days 1,8,15	300	68	[3, 7, 13]
$Aminopterin_{po}$	2 mg/m^2 (po) q12h ×2 days 1,8,15	12	6	[7]
$Methotrexate_{iv}$	100–200–300 mg/m^2 (iv) days 1,8,15	600	220	[3, 20]
$Talotrexin_{iv}$	54 mg/m^2 (iv) days 1,8	108	78	*
$Pemetrexed_{iv}$	1910 mg/m^2 (iv) day 1	1910	1762	[19]

Total dose and AUC data are cumulative for doses administered over a 21-day cycle

* Personal communication G. Berk

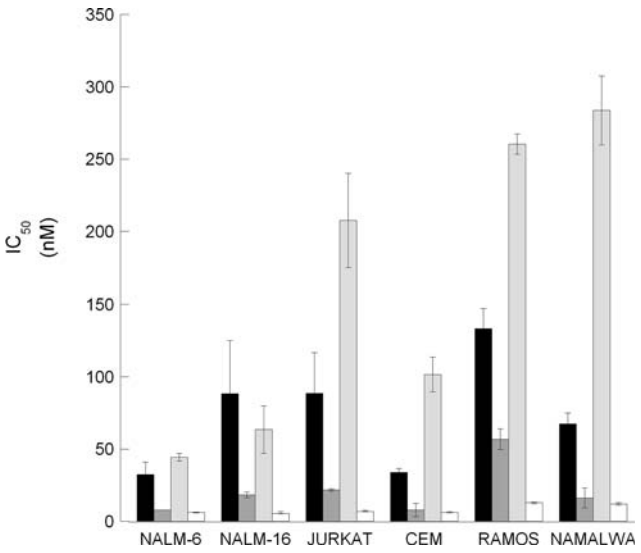


Fig. 2 IC_{50} of methotrexate (*black*), aminopterin (*dark gray*), pemetrexed (*light gray*), and talotrexin (*white*) in childhood leukemia and lymphoma cell lines. Standard deviation depicted by error bars. Talotrexin followed by aminopterin had the lowest IC_{50}'s

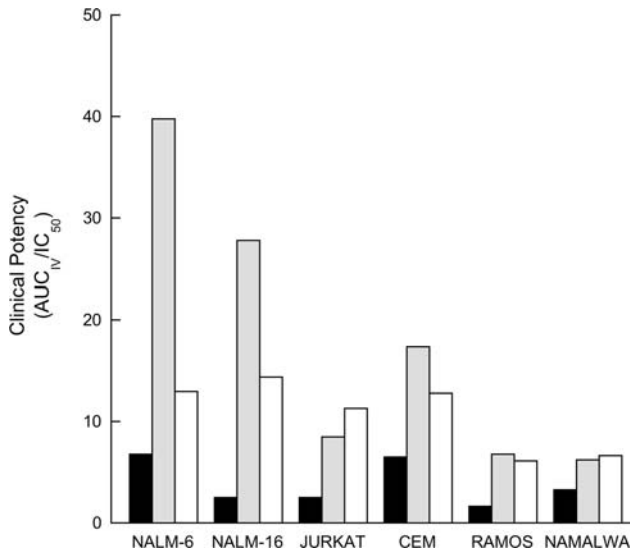


Fig. 3 CPI of pemetrexed (*light gray*) and talotrexin (*white*) compared to methotrexate (*black*) using IV schedule of methotrexate. Pemetrexed and talotrexin had a higher CPI than methotrexate

MTX: 25 mg/m^2 every 6 h for four doses [7]), MTX appears to be the more effective antifol. These results suggest AMT is unlikely to be more efficacious than MTX in the treatment of children with leukemia.

Talotrexin is a structurally novel antifol that combines characteristics of both classical and non-classical antifols. Like aminopterin, its nucleus is formed by a substitution of an amino group for the hydroxyl group at the 4-position of on the pteridine ring of folic acid. It does not, however, have a glutamic acid side chain and thus cannot be

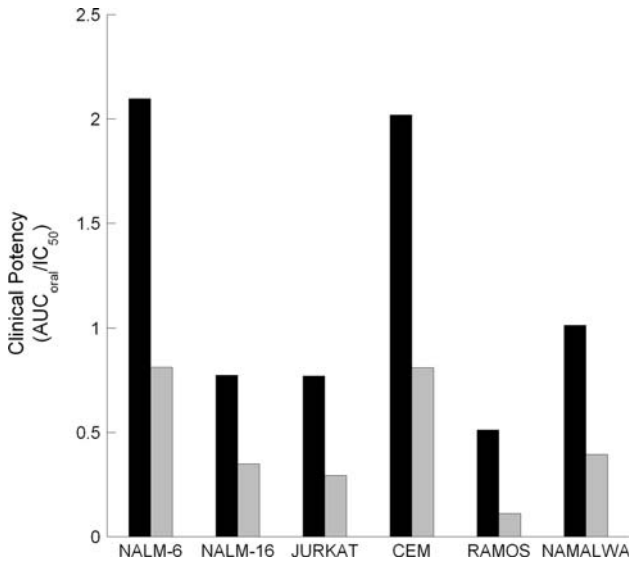


Fig. 4 CPI of aminopterin (*gray*) compared to methotrexate (*black*) using oral schedule of methotrexate. Methotrexate had a higher CPI than aminopterin in all cell lines

polyglutamated. Relative to methotrexate, talotrexin is transported tenfold more efficiently by the membrane-bound RFC [26, 34], it is tenfold more tightly bound to the target enzyme DHFR [16] and it is 5–100 fold more potent in a wide variety of pre-clinical models [25, 26]. Unfortunately, unexpected central nervous system toxicity at higher dose levels has limited further clinical development of this antifol [33].

With respect to new antifols, pemetrexed is the most advanced in progressing through pediatric clinical development. Pemetrexed is a structurally novel antifol that posses a unique 6,5 fused pyrrolo [2,3-d] pyrimidine nucleus but maintains glutamic acid side chain thus allowing for polyglutamation [32]. Pemetrexed enters the cell through the reduced folate carrier (RFC) and membrane folate binding protein transport systems. It is a substrate for multidrug resistance protein transporters and is polyglutamated in a reaction catalyzed by folylpolyglutamate synthase. When compared with methotrexate, pemetrexed is polyglutamated 90–200 fold more efficiently [29]. It inhibits thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT), but unlike methotrexate and aminopterin, its primary target is TS. These differences in target enzyme affinities suggest that pemetrexed could demonstrate activity in diseases that have either de novo or acquired resistance to methotrexate.

This analysis demonstrates a novel approach of incorporating human clinical pharmacologic data to produce a more integrated interpretation of in vitro cytotoxicity experiments. We believe that utilizing clinically achievable drug exposure data will help to prioritize agents with the

potential for clinical efficacy that may not have been detected by in vitro analysis alone and will help to identify compounds whose potential for in vivo efficacy may be limited by clinical tolerability. When our in vitro cytotoxicity results were analyzed using the CPI, aminopterin did not appear to have an advantage over methotrexate, but pemetrexed did have a greater CPI than methotrexate in all cell lines. These results support pursuing additional studies of pemetrexed for childhood ALL.

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