DE NOVO PURINE SYNTHESIS INHIBITION AND ANTILEUKEMIC EFFECTS OF MERCAPTOPURINE ALONE OR IN COMBINATION WITH METHOTREXATE, IN VIVO


a Department of Pharmaceutical Sciences, b Department of Biostatistics, c Department of Hematology-Oncology, d Department of Pathology;
St. Jude Children's Research Hospital, Memphis 38105 TN, USA;

University of Tennessee, Memphis, 38103 TN, USA. * These authors contributed equally.

Research grant support: This work was supported by grants CA 36401, CA 78224, CA21765 and CA51001 from the National Institutes of Health; by a Center of Excellence grant from the State of Tennessee; and by American Lebanese Syrian Associated Charities (ALSAC).

Running title: Effects of antimetabolites on de novo purine synthesis.

Scientific heading: Clinical observation, intervention, and therapeutic trials.

Word count: Abstract: 250 words; Manuscript: 4045

Correspondence:

Dr William Evans
Department of Pharmaceutical Sciences
St Jude Children's Research Hospital,
332 North Lauderdale
Memphis, 38105 TN.

Phone: 901-495-3663; Fax: 901-525-6869; E-mail: william.evans@stjude.org
ABSTRACT

Methotrexate (MTX) and mercaptopurine (MP) are widely used antileukemic agents that inhibit de novo purine synthesis (DNPS) as a mechanism of their antileukemic effects. To elucidate pharmacodynamic differences among children with acute lymphoblastic leukemia (ALL), DNPS was measured in leukemic blasts from newly diagnosed patients, before and after therapy with these agents. Patients were randomized to receive low-dose MTX (LD: 6 oral doses of 30 mg/m²) or high-dose MTX (HD: IV 1 g/m²) followed by IV MP; or IV MP alone (1 g/m²), as initial therapy. At diagnosis, the rate of DNPS in bone marrow leukemia cells was 3-fold higher in patients with T-lineage ALL compared to those with B-lineage ALL (769±189 versus 250±38 fmol/nmol/h; p=0.001). DNPS was not consistently inhibited following MP alone, but was markedly inhibited following MTX+MP (median decrease= 3% versus 94%; p<0.001). LDMTX+MP and HDMTX+MP produced greater antileukemic effects (percentage decrease in circulating leukocyte counts) compared to MP alone (-50±4%, -56±3%, and -20±4%, respectively; p<0.0001). Full DNPS inhibition was associated with greater antileukemic effects compared to partial or no inhibition (-63±4% versus -37±4%; p<0.0001) in patients with non-hyperdiploid B-lineage and T-lineage ALL. HDMTX+MP yielded 2.0-fold higher MTX polyglutamate concentrations than LDMTX+MP (2148±298 versus 1075±114 pmol/10⁹ cells; p<0.01), and a higher percentage of patients with full DNPS inhibition (78% versus 53%; p<0.001). Thus, the extent of DNPS inhibition was related to in vivo antileukemic effects, and a single dose of IV MP produced minimal DNPS inhibition and antileukemic effects, whereas MTX+MP produced greater antileukemic effects and DNPS inhibition, with full inhibition more frequent after HDMTX.
INTRODUCTION

Methotrexate and mercaptopurine are antimetabolites that form the cornerstone of continuation therapy for childhood acute lymphoblastic leukemia (ALL). Methotrexate and mercaptopurine are antimetabolites that form the cornerstone of continuation therapy for childhood acute lymphoblastic leukemia (ALL). Both of these agents can inhibit de novo purine synthesis (DNPS), which is postulated as a mechanism of their antileukemic effects, and a rationale for using them in combination. Methotrexate is converted by folylpolyglutamate synthase to methotrexate polyglutamates (MTXPGs). MTXPGs inhibit dihydrofolate reductase, thereby decreasing the amount of reduced folates, the one-carbon donor for the purine ring formation in DNPS, and MTXPGs also directly inhibit phosphoribosylpyrophosphate (PRPP)-amidotransferase, glycinamide ribonucleotide transformylase and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase, key enzymes in the DNPS pathway. Mercaptopurine is metabolized by the purine salvage enzyme, hypoxanthine-guanine phosphoribosyltransferase (HPRT), to thioinosine monophosphate, and subsequently metabolized to thioguanine nucleotides, or alternatively metabolized by thiopurine methyltransferase to methylthioinosine monophosphate, an inhibitor of PRPP-amidotransferase. The consequence of DNPS inhibition by these antimetabolites is purine deprivation, leading to inhibition of DNA synthesis, decreased cell proliferation, and cytotoxicity.

Although in vitro studies have demonstrated that DNPS inhibition by methotrexate and/or mercaptopurine contributes to their cytotoxic effects, and that methotrexate putatively acts synergistically with mercaptopurine by enhancing the accumulation of PRPP, a necessary co-
factor for mercaptopurine activation,\textsuperscript{9,10} these mechanisms have not been established in primary leukemia cells, \textit{in vivo}.

In the present investigation, we determined the effects of a single dose of mercaptopurine alone or in combination with low dose or high dose methotrexate, on DNPS in bone marrow leukemia cells from patients with newly diagnosed ALL. These studies revealed significant lineage-differences in DNPS rates at diagnosis of ALL and a significant relation between the extent of DNPS inhibition and antileukemic effects of these medications.
METHODS

Patients and treatment

Children aged 18 or younger with newly diagnosed acute lymphoblastic leukemia (ALL) were enrolled on the TOTAL XIIIB protocol from 1994 to 1998. The study was approved by the Institutional Review Board at St Jude Children’s Research Hospital. Signed informed consent was obtained from parents or legal guardians before enrollment in the protocol. The diagnosis of ALL was based on morphology, cytochemical staining properties and immunophenotyping of blast cells for classification as B-lineage or T-lineage, as previously described. Ploidy was determined based on DNA index (DI: ratio of DNA content in leukemic cells versus normal diploid G0/G1 cells), and classified as non-hyperdiploid or hyperdiploid. Mature B-cell ALL cases were excluded.

After stratification for age, leukocyte count, immunophenotype and sex, patients were randomized to receive one of three up-front therapies (Figure 1): intravenous mercaptopurine alone (IV MP: 1 g/m² over 6 hours consisting of 200 mg/m² over 20 minutes and 800 mg/m² over 5 hours 40 minutes); low dose oral methotrexate (LDMTX: 30 mg/m² every 6 hours for a total of 6 doses) followed by IV MP (same dose as above); or high dose intravenous methotrexate (HDMTX: 1 g/m² over 24 hours followed by IV mercaptopurine (1 g/m²) over 6 hours (HDMTX+MP). De novo purine synthesis rates were measured in leukemia cells from bone marrow (BM) aspirates obtained at diagnosis and at 20 hours after the start of mercaptopurine infusion (corresponding to 44 hours after the start of methotrexate therapy). Methotrexate polyglutamate concentrations were measured in bone marrow leukemia cells obtained at 44 hours after initiation of chemotherapy in patients randomized to methotrexate plus mercaptopurine.

Figure 1: Treatment schema. Patients with newly diagnosed ALL were randomized to one of three treatments: IV mercaptopurine (1 g/m²) over 6 hours (MP); or oral methotrexate (30 mg/m² every 6 hours, 6 doses) followed by 24 hours by IV mercaptopurine (1 g/m²) over 6 hours (LDMTX+MP); or intravenous methotrexate (1 g/m²) over 24 hours followed by IV mercaptopurine (1 g/m²) over 6 hours (HDMTX+MP). De novo purine synthesis rates were measured in leukemia cells from bone marrow (BM) aspirates obtained at diagnosis and at 20 hours after the start of mercaptopurine infusion (corresponding to 44 hours after the start of methotrexate therapy). Methotrexate polyglutamate concentrations were measured in bone marrow leukemia cells obtained at 44 hours after initiation of chemotherapy in patients randomized to methotrexate plus mercaptopurine.
(HDMTX: 1 g/m^2 over 24 hours consisting of 200 mg/m^2 IV push and 800 mg/m^2 over 24 hours) immediately followed by IV MP (same as above). Intravenous methotrexate and oral methotrexate were purchased from Lederle, Laboratories (Pearl River, NY). Intravenous mercaptopurine (Purinethiol) was supplied by the National Cancer Institute as a lyophilized powder of sodium salt of mercaptopurine (0.5 mg per vial). Mercaptopurine salt was reconstituted with sterile water for injection. Patients received hydration with intravenous dextrose 5%/0.25 normal saline with 40 meq NaHCO_3/liter. NaHCO_3 was given as needed to maintain urine pH \geq 6.5 but <8.0. All patients treated with methotrexate received leucovorin rescue (10 mg/m^2 orally or intravenously every 6 hours for 5 doses starting 48 hours from the start of methotrexate). Leucovorin rescue was continued until the plasma methotrexate concentration was below 0.1 \mu M.

To prevent or treat hyperuricemia and tumor lysis syndrome, urate oxidase^{13} (Uricozyme, Sanofi-Synthelabo, Paris, France) or its recombinant analogue (Rasburicase, Sanofi-Synthelabo, Paris, France) were administered intravenously if required. In case of contraindication to Uricozyme and Rasburicase (glucose 6-phosphate dehydrogenase deficiency, asthma, and history of atopic allergy), allopurinol (Zyloprim, Glaxo-wellcome) was given orally.

**Bone Marrow Collection and Processing**

A bone marrow aspirate (5-10 ml in a syringe containing 800 U heparin) was obtained at 20 hours after initiation of the mercaptopurine infusion. It was kept on ice, diluted with HHH
solution (Hank’s, heparin and HEPES) and bone marrow lymphoblasts were isolated on a Ficoll density gradient, as previously described.\textsuperscript{14}

**Evaluation of response to chemotherapy**

Circulating leukocyte counts were measured before therapy (day 0) and at day 3, prior to the administration of other antileukemic agents. Leukocyte counts were determined with a Coulter counter (model F+STKR; Coulter Corp., Hialeah, FL). The percentage change in leukocyte count 3 days after beginning chemotherapy was determined as the day 3 count minus the day 0 count, divided by the day 0 count, and multiplied by 100.

**Determination of bone marrow purine bases, rate of de novo purine synthesis (DNPS) and methotrexate polyglutamate concentrations**

The concentration of purine bases from hydrolyzed nucleotides and the rate of DNPS in ALL blasts were simultaneously determined by quantifying unlabelled and radiolabeled purine bases (adenine and guanine) after acid hydrolysis of a 2 hour \textit{ex vivo} incubation of $5 \times 10^6$ lymphoblasts with $^{14}$C-formate, as previously described.\textsuperscript{14,15} The concentrations of unlabelled adenine and guanine were determined from peak areas of UV chromatograms against a linear calibration curve of standards (1-75 nmol on column) prepared in phosphate buffered saline. Newly synthesized purines were determined by the concentration of $^{14}$C-adenine and $^{14}$C-guanine, which were calculated based on the total d.p.m. corresponding to the relevant peaks of the chromatograms, with a specific activity of 50 d.p.m./pmol. The detection limit for the
radiolabeled purines was 0.02 pmol on column. The rate of *de novo* adenine plus guanine synthesis (DNPS) was calculated as the fmol of newly synthesized adenine plus guanine per nmol of total intracellular adenine plus guanine per hour of incubation (fmol/nmol/h). Under conditions used, the rate of DNPS (14C incorporation from formate) remained linear for incubation times of 30 to 120 minutes in human CEM-CCRF leukemia cells (linear correlation coefficient = 0.986±0.009, mean of three experiments). Similarly, the rate of *de novo* adenine or *de novo* guanine synthesis was calculated as the fmol of newly synthesized adenine or guanine per nmol of total intracellular adenine plus guanine per hour of incubation. The percentage change in DNPS 20 hours after initiation of mercaptopurine was determined by comparison with the initial DNPS (subtraction of the 20 hours DNPS from the pre-therapy DNPS, dividing by the pre-therapy DNPS, and multiplying by 100). Intracellular purine concentrations are expressed as nmol per 10^6 cells. Inhibition of DNPS was classified as full (>90%), partial (10-89%) or no (<10%) inhibition. The HPLC separation and measurement of methotrexate and six polyglutamated metabolites (MTXPG2 to MTXPG7) were performed as previously described.16

**Statistical Analyses**

The distributions of gender, immunophenotypes, ploidy, type of uricolytic therapy received in the 72 h preceding key time points (bone marrow aspirates at diagnosis and after chemotherapy), as well as the patterns of DNPS inhibition were assessed with either the chi-square or exact chi-square test as appropriate. Quantitative variables and demographic characteristics were compared among the three treatment arms with the exact Kruskal-Wallis test, or with the exact Wilcoxon Rank Sum test17 when only two treatment groups were considered. Change between
any two time points was assessed with the exact Wilcoxon Sign Rank test. No adjustment have been made for multiple testing. Results are expressed as mean ± standard error, unless otherwise indicated.
RESULTS

Patients

Between August 1994 and July 1998, 233 children with newly diagnosed ALL were enrolled on the St. Jude Total XIIIB protocol and randomized to receive one of three initial treatments; MP alone (76 patients), LDMTX + MP (83 patients), or HDMTX + MP (74 patients). There were no significant differences in demographic characteristics (Table I) or the frequency of uricolytics administered among the three groups of patients, or between patients randomized to MP alone versus MTX+MP.

Demographics of patients randomized to MP alone or LDMTX+MP or HDMTX+MP.

<table>
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<tr>
<th>Demographic</th>
<th>MP alone (n=76)</th>
<th>LDMTX + MP (n=83)</th>
<th>HDMTX + MP (n=74)</th>
<th>Inter-group comparison p value</th>
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<tr>
<td>Age (yr)</td>
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<td>5.9 (0.5-17.1)</td>
<td>5.9 (0.3-18.8)</td>
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<tr>
<td>WBC day 0 (x10^3/mm^3)</td>
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<td>7.5 (1-399)</td>
<td>7.4 (1-276)</td>
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</table>

Number of patients with uricolytics administered within 72 hours prior to:

Bone marrow diagnosis

Urate oxidase
Allopurinol or with urate oxidase
None

Bone marrow at 20 hours after start of MP

Urate oxidase
Allopurinol alone or with urate oxidase
None

All results are as median (range) for quantitative variables.
Effect of chemotherapy among treatment regimens and immunophenotypes

At initiation of chemotherapy, leukocyte counts were similar among the three treatment groups (p=0.83; n=233), whereas within each treatment arm, patients presenting with T-lineage ALL had higher leukocyte counts than those with B-lineage ALL (p<0.001). As depicted in Figure 2A, patients with non-hyperdiploid B-lineage ALL presented with higher leukocyte counts than those with hyperdiploid B-lineage ALL (p<0.001).

Antileukemic response was evaluable in 195 patients (38 were not evaluable because leukocyte count was not determined on day 3), based on the decrease in circulating leukocytes over the initial three days of treatment. As depicted in Figure 2B, the average percentage decrease in leukocyte counts was significantly less in patients randomized to MP alone (-20±4%, n=51) compared to patients randomized to LDMTX+MP (-49±4%, n=77) or HDMTX+MP (-56±4%, n=67) (p<0.001). Patients in the HDMTX+MP group tended to have a greater percentage decrease in leukocyte counts than those in the LDMTX+MP group, but the difference was not
statistically significant (p=0.33). Similarly, when the analysis was restricted to each of the three leukemic subtypes (hyperdiploid or non-hyperdiploid B-lineage ALL or T-lineage ALL), methotrexate plus mercaptopurine produced greater antileukemic effects than MP alone (p<0.004), but no significant difference in the antileukemic response was observed between the two methotrexate treatment regimens (p>0.24).

In patients randomized to MP alone, a similar percentage decrease in leukocyte counts was observed among the three leukemic subtypes (p=0.137) (Figure 2B). In patients who received methotrexate (high dose or low dose) plus mercaptopurine, those with T-lineage ALL had a greater percentage decrease in leukocyte counts (-69±6%, n=22) than those with non-hyperdiploid B-lineage ALL (-55±4%, n=80; p=0.075) or hyperdiploid B-lineage ALL (-40±5%, n=41; p<0.001). Also, patients with non-hyperdiploid B-lineage ALL had a greater percentage decrease in leukocyte counts than those with hyperdiploid B-lineage ALL (p=0.004).

**De novo purine synthesis and intracellular purine concentrations**

At diagnosis, bone marrow DNPS rates and intracellular purine concentrations were evaluable in 194 patients (40 were not evaluable because of poor yields of the bone marrow aspirate). No significant effect of uricolytics (within 72 hours prior to the bone marrow aspirate) was observed on *de novo* adenine, guanine, or adenine plus guanine synthesis rates (p>0.20). *De novo* adenine, guanine, and *de novo* adenine plus guanine synthesis rates were higher in T-lineage ALL (n=32) than B-lineage ALL (n=161), with no difference between hyperdiploid B-lineage ALL (n=55) and non-hyperdiploid B-lineage ALL (n=106) (p>0.60) (Figure 3A). Patients with T-lineage
ALL had on average 4-fold and 2-fold higher *de novo* adenine and *de novo* guanine synthesis rates, when compared to B-lineage ALL (p=0.0007 and p=0.010 respectively), resulting in a 3.0-fold higher total DNPS rate (adenine plus guanine) in T-lineage ALL compared to B-lineage ALL (769±189 versus 250±38 fmol/nmol/h; p=0.001) (Figure 3A).

Bone marrow hypoxanthine plus inosine concentrations were higher in hyperdiploid B-lineage ALL (1.10±0.29 nmol/10⁶ cells, n=42) than in non-hyperdiploid B-lineage ALL (0.65±0.12 nmol/10⁶ cells, n=93), and higher in hyperdiploid B-lineage ALL than T-lineage ALL (0.41±0.20 nmol/10⁶ cells, n=27) (p<0.001). Furthermore, adenine concentrations were higher in hyperdiploid B-lineage ALL (5.10±0.37 nmol/10⁶ cells, n=51) than in non-hyperdiploid B-lineage ALL (4.16±0.26 nmol/10⁶ cells, n=99), and higher than in non-hyperdiploid B-lineage ALL compared to T-lineage ALL (3.64±0.24 nmol/10⁶ cells, n=27) (p=0.007). However, intracellular guanine concentrations were similar among the three leukemic subtypes (p=0.52) (Figure 3B).
Effect of treatment regimens on de novo purine synthesis

The change in DNPS rate from initiation of chemotherapy to 20 or 44 hours after chemotherapy was evaluable in 158 patients (51 patients randomized to MP, 62 patients to LDMTX+MP and 45 to HDMTX+MP). The remaining 75 patients were not evaluated because there were insufficient cells to measure DNPS at diagnosis or after treatment. In patients randomized to mercaptopurine alone, there was no significant inhibition of DNPS rate at 20 hours (median change of -3%, n=51; p=0.34). In contrast, as depicted in Figure 4A, there was inhibition of DNPS in patients randomized to receive LDMTX+MP (median change of -94%, n=62; p<0.001) or HDMTX+MP (median change of -99%, n=45; _p<0.001). These differences were similar when either de novo adenine or de novo guanine synthesis rates were compared (not shown).

Figure 4: Effect of treatment regimens on de novo purine synthesis.
Panel A: In patients randomized to mercaptopurine (n=51), DNPS rate was not significantly inhibited (p=0.34), whereas inhibition was evident in patients randomized to low dose methotrexate (p<0.01; n=62) or high dose methotrexate (p<0.01; n=45).
Panel B: A lower frequency of full DNPS inhibition was achieved in patients who received mercaptopurine compared to methotrexate plus mercaptopurine (20% versus 68%; p<0.01). High dose methotrexate with mercaptopurine produced full inhibition in a greater percentage of patients, compared to low dose methotrexate plus mercaptopurine (78% versus 53%; p=0.03).
Patients with hyperdiploid B-lineage ALL exhibited less inhibition of DNPS (median change of -4%, n=43) compared to those with non-hyperdiploid B-lineage ALL (median change -94%, n=85; p=0.004) or T-lineage ALL (median change -94%, n=30; p=0.017). However, no difference in the inhibition of DNPS rate was observed between T-lineage ALL and non-hyperdiploid B-lineage ALL (p=0.93).

Among 158 evaluable patients, full DNPS inhibition occurred in 78 patients (49%), partial inhibition in 47 patients (30%) and no inhibition in 33 patients (21%). The percentage of patients having full DNPS inhibition was significantly lower after MP alone (20%) compared to LDMTX+MP (53%; p<0.001) or HDMTX+MP treatment (78%; p<0.001). Furthermore, full DNPS inhibition was achieved in a higher percentage of patients randomized to HDMTX+MP compared to LDMTX+MP (p=0.027) (Figure 4B).

**Effects of methotrexate polyglutamates on de novo purine synthesis**

In 106 patients treated with methotrexate plus mercaptopurine, methotrexate polyglutamate concentrations were measured in leukemia cells from bone marrow aspirates obtained 44 hours after the start of methotrexate treatment. Patients randomized to HDMTX+MP had on average 2.0-fold higher methotrexate polyglutamate concentrations (MTXPG\(_{1-7}\): 2148±298 pmol/10\(^9\) cells, n=47) than those randomized to LDMTX+MP (1075±114 pmol/10\(^9\) cells, n=59) (p=0.0027). Similarly, HDMTX+MP treatment produced 1.7-fold higher long-chain methotrexate polyglutamate concentrations (MTXPG\(_{4-7}\): 1316±159 pmol/10\(^9\) cells, n=47) compared to LDMTX+MP (818±88 pmol/10\(^9\) cells, n=59) (p=0.0141).
As depicted in Figure 5A, patients with T-lineage ALL had significantly lower MTXPG\textsubscript{1-7} concentrations (691±146 pmol/10\textsuperscript{9} cells, n=23) than patients with non-hyperdiploid B-lineage ALL (1467±161 pmol/10\textsuperscript{9} cells, n=61; p<0.001), and patients with non-hyperdiploid B-lineage ALL had lower MTXPG\textsubscript{1-7} concentrations than those with hyperdiploid B-lineage ALL (2684±499 pmol/10\textsuperscript{9} cells, n=22; p=0.001). Similar results were observed when long chain MTXPG\textsubscript{4-7} concentrations were compared (Figure 5B) or when lineage and ploidy were assessed within the LDMTX+MP or the HDMTX+MP groups. The administration of HDMTX+MP resulted in 2.1-fold higher MTXPG\textsubscript{1-7} concentrations compared to LDMTX+MP administration in patients with hyperdiploid B-lineage ALL (4033±1005 pmol/10\textsuperscript{9} cells, n=9 versus 1751±308 pmol/10\textsuperscript{9} cells, n=13; p<0.01) or non-hyperdiploid B-lineage ALL (2043±284 pmol/10\textsuperscript{9} cells, n=28 versus 978±127 pmol/10\textsuperscript{9} cells, n=33; p<0.01). However, in patients with T-lineage ALL, the administration HDMTX+MP did not produce
higher MTXPG$_{1-7}$ concentrations when compared to LDMTX+MP (749±274 pmol/10$^9$ cells, n=10 versus 646±161 pmol/10$^9$ cells, n=13, respectively; p=0.49) (Figure 5A).

There were significantly higher MTXPG$_{1-7}$ concentrations in leukemia cells of patients with full inhibition of DNPS (1803±236 pmol/10$^9$ cells, n=52) compared to patients with partial or no inhibition (939±124 pmol/10$^9$ cells, n=31) (p=0.0172) (Figure 5C). Similar results were observed when only patients with hyperdiploid or non-hyperdiploid B-lineage ALL were considered (p<0.025); however, within patients with T-lineage ALL, there was not a statistically significant difference, although the trend was similar (p=0.35) (Figure 5C). Significantly lower MTXPG$_{1-7}$ concentrations were required for full DNPS inhibition in patients with T-lineage ALL (831±215 pmol/10$^9$ cells, n=14) compared to those with non-hyperdiploid B-lineage ALL (1845±303 pmol/10$^9$ cells, n=28) or hyperdiploid B-lineage ALL (3044±674 pmol/10$^9$ cells, n=10) (p=0.001). Similar differences were observed with long chain MTXPG$_{4-7}$ (Figure 5D), or when the analysis was restricted to the LDMTX+MP or the HDMTX+MP group (data not shown).

**Effect of treatment regimen on de novo purine synthesis and relationship to response to chemotherapy**
Among the 158 patients with evaluable DNPS inhibition, 135 (85%) were also evaluable for initial response to chemotherapy. As depicted in Figure 6A, full DNPS inhibition was associated with a significantly greater percentage decrease in circulating leukocytes (-57±4%, n=71) compared to partial inhibition (-38±7%, n=38; p=0.008) or no inhibition (-29±6%, n=26; p<0.001). However, there was no significant difference in the percentage decrease in circulating leukocytes between patients having partial or no inhibition (p=0.11). Therefore, these two groups (47% of patients) were combined and compared to the group of patients (53%) having full DNPS inhibition.

In patients with non-hyperdiploid B-lineage ALL or T-lineage ALL, those having full inhibition of DNPS had a greater percentage decrease in circulating leukocytes compared to those with partial or no inhibition (B-lineage; -59.9%±5.0, n=39 versus -37.8%±6.2 n=36, p=0.004; and T-lineage; -70.6%±7.2, n=15 versus -33.8%±10.6, n=8; p=0.02, respectively). In contrast, no such difference was evident in patients with hyperdiploid B-lineage ALL (-36.8%±7.8, n=17 versus -
28.8%±7.0, n=20 for full vs. partial/no inhibition, respectively, p=0.43) (Figure 6B). In patients having partial or no DNPS inhibition, no differences in the percentage decrease of circulating leukocyte were observed among lineage or ploidy subgroups (p=0.40). In contrast, in patients having full DNPS inhibition, non-hyperdiploid B-lineage and T-lineage ALL had a greater percentage decrease in circulating leukocytes counts compared to patients with hyperdiploid B lineage ALL (p<0.01).

Within patients randomized to methotrexate plus mercaptopurine, those with hyperdiploid B-lineage ALL did not exhibit a significantly greater percentage decrease in circulating leukocyte counts with full DNPS inhibition versus partial or no inhibition of DNPS (-36±8%, n=16 versus -52±9, n=9 respectively, p=0.22). These findings were similar within the LDMTX+MP or the HDMTX+MP groups (data not shown).

Within patients with non-hyperdiploid B-lineage or T-lineage ALL randomized to LDMTX+MP (n=42) those with full DNPS inhibition had a greater percentage decrease in leukocyte counts (-67±6%, n=22) compared to those with partial or no inhibition (-37±11%, n=20) (p=0.005). In contrast, within the HDMTX+MP treatment group (n=31), the percentage decrease in circulating leukocyte count did not differ in patients with full versus partial or no DNPS inhibition (-72±11%, n=26 versus -67±4%, n=5; p=0.55), suggesting additional or alternative mechanisms of cytotoxicity with HDMTX. Interestingly, within the largest lineage and ploidy group (patients with non-hyperdiploid B-lineage ALL, about 70% of childhood ALL), in patients with partial or no DNPS inhibition, HDMTX+MP produced a greater percentage decrease (p=0.032) in leukocyte counts (-72±11%, n=5) than LDMTX+MP (-36±13%, n=16). However, when there
was full DNPS, there was no difference in antileukemic effects between LDMTX+MP and HDMTX+MP (-65±5%, n=18 with HDMTX+MP versus -61±8%, n=16 with LDMTX+MP; p=0.98).
DISCUSSION

We have investigated in a randomized clinical trial the \textit{in vivo} antileukemic effects of mercaptopurine alone or in combination with low dose or high dose methotrexate in children with ALL. These two agents are among the most widely used medications in the curative therapy of childhood ALL, yet their mechanisms and optimal doses remain to be fully elucidated in different ALL subtypes. Previous studies suggested that high-dose intravenous mercaptopurine plus high-dose methotrexate improve outcome in childhood ALL\textsuperscript{18,19}. However, the therapeutic effect of high-dose mercaptopurine alone has not been evaluated. The objective of the current research was to determine whether mercaptopurine alone or in combination with methotrexate inhibits DNPS and how this relates to the antileukemic effects of these medications.

Purine nucleotides are synthesized by the salvage pathway and by the \textit{de novo} pathway, the latter being specifically measured in the current study. We found that leukemic cells from patients with T-lineage ALL have on average 4-fold and 2-fold higher \textit{de novo} adenine and \textit{de novo} guanine synthesis rates at diagnosis, resulting in a 3.0-fold higher total DNPS rate when compared to patients with B-lineage ALL. In addition, patients with T-lineage ALL had lower intracellular concentrations of inosine, hypoxanthine and adenine, but similar guanine concentrations compared to those with B-lineage ALL. These new findings are consistent with prior studies reporting constitutive differences in purine enzyme activities in T-lineage ALL compared to B-lineage ALL\textsuperscript{20,21}. Therefore, T-lineage ALL may be more dependent on the \textit{de novo} pathway for purine synthesis (especially adenine) compared to B-lineage ALL, a finding consistent with
previous *in-vitro* data in cell lines,⁹ and with recent evidence²²-²⁴ that methylthioadenosine phosphorylase (an adenine salvage gene) is more frequently deleted in T-lineage than in B-lineage ALL, because of its close genomic proximity to the tumor suppressors P16⁴⁴A/P15⁴⁴B. In addition, hypoxanthine inhibits DNPS by feed-back mechanisms,²⁵,²⁶ and the lower intracellular hypoxanthine plus inosine concentrations in T-lineage ALL compared to B-lineage ALL suggests that lower purine recycling and salvage activity may be compensated by increased DNPS in T-lineage ALL.

*In vitro*, methotrexate produces DNPS inhibition through folate depletion as well as direct inhibition by methotrexate polyglutamates of key DNPS enzymes, amidophosphoribosyltransferase, AICAR and GAR transformylases.³-⁵ In contrast, mercaptopurine is known to inhibit DNPS only through inhibition of amidophosphoribosyltransferase by methylthioinosine nucleotides.⁷ Our data establish that a single high-dose of mercaptopurine does not consistently inhibit DNPS in ALL cells *in vivo*, whereas the combination of methotrexate and mercaptopurine does. It is unclear whether inhibition of DNPS is due predominantly to methotrexate, but this is likely because mercaptopurine had little effect alone and high dose methotrexate produced greater inhibition than low dose methotrexate. In this regard, our data support the use of high dose methotrexate in childhood ALL, to more consistently achieve full DNPS inhibition, compared to low dose methotrexate, because full DNPS inhibition produced greater antileukemic effects compared to partial or no inhibition.

Partial DNPS inhibition was not associated with significantly greater antileukemic effects when compared to no DNPS inhibition, suggesting that full DNPS inhibition is required to trigger cell
death. This is consistent with the notion that leukemia cells rely more on the de novo pathway, than on salvage mechanisms, to synthesize purines, and that near complete inhibition of purine synthesis may be required to produce a purine-less state, leading to cell death. However, DNPS is only one mechanism of action shared by methotrexate and mercaptopurine, and a component of the antileukemic effects of these two agents may be mediated via alternative mechanisms, such as inhibition of thymidylate synthase by methotrexate polyglutamates or incorporation of deoxythioguanosine into DNA following mercaptopurine. It is known that methotrexate polyglutamates inhibit other targets in a concentration-dependent manner, and the current work further establishes that high dose methotrexate achieves higher methotrexate polyglutamates concentrations in ALL blasts in vivo, compared to low dose methotrexate.

Interestingly, the relationship of DNPS inhibition to antileukemic effect was dissimilar among leukemic subtypes. In patients with non-hyperdiploid B-lineage and T-lineage ALL, full DNPS inhibition produced greater antileukemic effects than partial or no inhibition, whereas in patients with hyperdiploid B-lineage ALL, no relationship was evident between the extent of DNPS inhibition and antileukemic response. This suggests that DNPS inhibition may be a more important mechanism for antileukemic effects in patients with non-hyperdiploid B-lineage or T-lineage ALL, than hyperdiploid B-lineage ALL. Unexpectedly, patients with T-lineage ALL had a greater decrease in circulating leukocytes than those with B-lineage ALL following methotrexate. It is plausible that T-lineage ALL is more susceptible to DNPS inhibition than B-lineage ALL, because T-ALL blasts rely more on de novo purine synthesis than on the purine salvage pathway. In contrast, more efficient purine salvage or purine recycling mechanisms in hyperdiploid B-lineage (consistent with greater hypoxanthine and inosine
concentrations at diagnosis), may explain their lower sensitivity to DNPS inhibition, compared to non-hyperdiploid B-lineage ALL. A good initial response during the first week of chemotherapy has been associated with a good overall treatment outcome in patients with ALL (i.e. response to steroids). In that regard, results in the present study appear paradoxical, as patients with hyperdiploid B-lineage ALL exhibited lower initial response to chemotherapy compared to patients with T-lineage ALL, yet overall, patients with hyperdiploid B-lineage ALL have a better event free survival than most other ALL subtypes. However, it is possible that the initial decrease in leukocyte counts following methotrexate and mercaptopurine (over 72 hours in our study) does not have the same prognostic value as the steroid response over seven days.\textsuperscript{30} In addition, the improved event free survival in patients with hyperdiploid B-lineage ALL compared to patients with T-lineage ALL, might reflect their sensitivity to other chemotherapeutic agents given during 2.5-3 years of therapy (e.g., L-asparaginase and cytarabine).\textsuperscript{31}

In the LDMTX+MP group, patients with non-hyperdiploid B-lineage or T-lineage ALL had greater antileukemic response when full DNPS inhibition was achieved. In contrast, within the HDMTX+MP treatment group, the antileukemic response did not differ in patients with full versus partial or no DNPS inhibition. In addition, among patients with non-hyperdiploid B lineage ALL, HDMTX+MP produced a greater percentage decrease in leukocyte counts than LDMTX+MP in patients with partial or no inhibition of DNPS, and similar differences were observed between the two methotrexate arms in case of full inhibition. Taken together, these findings support the rationale for high dose methotrexate in childhood ALL, because HDMTX+MP is more likely to produce complete DNPS inhibition, and because HDMTX+MP
produces greater antileukemic effects in the absence of complete DNPS inhibition, consistent with additional mechanisms of action for HDMTX versus LDMTX. Going forward, it will be important to determine the optimal dosage and schedule of HDMTX in the major lineage and genetic subtypes of childhood ALL, to maximize the efficacy of this widely used antileukemic agent.
ACKNOWLEDGEMENTS

We thank our clinical staff for scrupulous attention to patient care and management of blood sampling; our research nurses, Sheri Ring, Lisa Walters, Terri Kuehner; and the patients and their parents for their participation in this study. We also thank Eve Su, YaQin Chu, May Chung, Kathryn Brown, Margaret Needham, Emily Melton, Anatoli Lenchik for technical assistance, and Nancy Kornegay for her computer and database expertise.
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DE NOVO PURINE SYNTHESIS INHIBITION AND ANTILEUKEMIC EFFECTS OF MERCAPTOPURINE ALONE OR IN COMBINATION WITH METHOTREXATE, IN VIVO

Thierry Dervieux, Timothy L Brenner, Yuen Y Hon, Yinmei Zhou, Michael L Hancock, John T Sandlund, Gaston K Rivera, Raul C Ribeiro, James M Boyett, C-H Pui, Mary V Relling and William E Evans

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