Gene expression and thioguanine nucleotide disposition in acute lymphoblastic leukemia after in vivo mercaptopurine treatment

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Abstract

To elucidate inter-patient variability in thioguanine nucleotide (TGN) concentrations in acute lymphoblastic leukemia (ALL) cells, we determined the TGN concentration in leukemic blasts from 82 children with newly diagnosed ALL after intravenous mercaptopurine (MP). Patients treated with MP alone, achieved higher TGN concentrations compared to those treated with the combination of methotrexate plus mercaptopurine (MTX+MP). Analysis of the expression of approximately 9,600 genes in ALL cells obtained at diagnosis identified 60 gene probes significantly associated with TGN accumulation in patients treated with MP alone and 75 gene probes in patients treated with MTX+MP, with no overlap between the two sets of genes. Genes significantly associated with intracellular TGN accumulation after MP alone, include those encoding MP metabolic enzymes and transporters (e.g., $SLC29A1$). Inhibition of $SLC29A1$ by nitrobenzylmercaptopurine ribonucleoside (NBMPR) caused a 33-45% reduction of TGN in ALL cells, in vitro ($p<0.006$), consistent with the gene expression findings. Genes associated with TGN concentration, after combination therapy include genes involved in protein and ATP-biosynthesis. Together, these in vivo and in vitro data provide new insights into the genomic basis of inter-patient differences in intracellular TGN accumulation, and reveal significant differences between treatment with MP alone or in combination with MTX.

Keywords: pharmacogenetics, gene expression, mercaptopurine, acute lymphoblastic leukemia
Introduction

Mercaptopurine (MP) is a purine antimetabolite widely used in the treatment of acute lymphoblastic leukemia (ALL)\(^1\). MP is an inactive prodrug that requires intracellular activation catalyzed by multiple enzymes, to exert cytotoxicity (Supplemental Figure A)\(^2\)-\(^5\). MP is converted by hypoxanthine guanine phosphoribosyltransferase (HGPRT) into thioinosine monophosphate (TIMP) and subsequently into thioguanosine monophosphate (TGMP) by a two-step process involving inosine monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthetase (GMPS)\(^2\). Subsequently, TGMP can be converted into the active thioguanine nucleotides (TGN) by adenosine kinase\(^2\)-\(^6\)-\(^7\). Cytotoxic effects of MP are achieved primarily through incorporation of TGN into RNA and DNA\(^8\)-\(^9\). The incorporation of TGN, as deoxy-TGN triphosphate, inhibits the function of several enzymes involved in DNA replication and repair\(^10\)-\(^11\) and induces DNA damage such as single-strand breaks, DNA-protein cross-links, interstrand cross-links and sister chromatid exchanges\(^12\)-\(^15\). The pathway leading to TGN synthesis is in competition with inactivation pathways catalyzed by xanthine oxidase (XDH) or thiopurine methyltransferase (TPMT)\(^16\).

Numerous clinical studies have reported substantial inter-patient variability in intracellular TGN concentrations during continuation therapy of acute lymphoblastic leukemia (ALL)\(^5\)-\(^17\)-\(^19\). Determinants of this variability include differences in MP absorption, genetic polymorphism of TPMT, and patient compliance to treatment\(^20\)-\(^23\). Inter-patient differences in intracellular TGN concentrations have been associated with treatment outcome in children with ALL, whereas variability in MP plasma concentrations has not. Patients with TGN accumulation above the population average had a better
relapse-free survival and long-term event-free survival (EFS) when treated with antimetabolite-based chemotherapy regimens\textsuperscript{5,24}.

In most treatment protocols for childhood ALL, MP is administered in combination with methotrexate (MTX). The pharmacological rationale for this combination is based in part on a direct effect of MTX on enzymes involved in TGN metabolism\textsuperscript{25} or indirectly through its effect on plasma purines\textsuperscript{17,26,27}. Recently, it has been shown that patients treated with this combination at a time when there are circulating ALL cells, accumulate lower intracellular TGN concentrations in bone marrow leukemic lymphoblasts, peripheral blasts, and erythrocytes when compared to patients who are treated with MP alone\textsuperscript{28}. However, there were substantial inter-individual differences in TGN accumulation in ALL cells following either treatment (MP alone: 0.21 to 16.6, MTX+MP: 0.08 to 1.74; 10\textsuperscript{th} to 90\textsuperscript{th} percentile range in pmol/5x10\textsuperscript{6} cells), for reasons that are largely unknown.

The present investigation was undertaken to identify genes significantly associated with accumulation of TGN in ALL cells in vivo, and to identify gene expression patterns that differed in leukemia cells that accumulated high versus low intracellular concentrations of TGN following administration of MP given alone or in combination with MTX.
Methods

Patients and treatment. A total of 82 children aged 18 years or younger with newly diagnosed ALL, enrolled on the St. Jude Children’s Research Hospital Total Therapy XIIIIB protocol, were included in the study. The investigation was approved by the Institutional Review Board and informed consent was obtained from patients, parents or legal guardians as appropriate. The diagnosis of ALL was based on morphology, cytochemical staining, immunophenotyping, cytogenetic analysis and genetic characterization, using methods previously described. Patients were randomized to receive one of three initial therapies (Supplemental Figure B). Patients received I.V. mercaptopurine alone (MP) (200 mg/m² over a 20-minute period plus 800 mg/m² over a period of 5 hours and 40 minutes); low-dose oral methotrexate (30 mg/m² every 6 hours for 6 doses) followed by I.V. mercaptopurine as above (LDMTX+MP); or high-dose I.V. methotrexate (200 mg/m² push plus 800 mg/m² over a 24-hour period) followed by I.V. mercaptopurine as above (HDMTX+MP). In cases of Uricozyme and Rasburicase contraindications, allopurinol (Zyloprim, Glaxo-Wellcome, Research Triangle Park, NC) was given orally. As previously described, use of allopurinol at least 72 hours prior to bone marrow aspiration did not alter thiopurine metabolite levels among treatment groups. For TGN analysis, we divided patients into three groups according to the randomized treatment arms (MP, n=33; HDMTX+MP, n=29; LDMTX+MP, n=20).

Isolation of ALL blasts from bone marrow aspirate. For all 82 patients, ALL cells at the time of diagnosis and 20 hours after initiation of the MP infusion (post-treatment) were obtained and processed as previously described. The percent of leukemic blasts in bone marrow aspirates at diagnosis and post-treatment was similar (median: 97% and 95%). The final cell yield was determined by hemocytometer and viability by trypan blue exclusion.
Determination of intracellular thiopurine nucleotide concentrations in bone marrow ALL cells. We used a reverse-phase high-performance liquid chromatography (HPLC) method reported previously to determine the amount of thioguanine nucleotides (TGN) in bone marrow cells 20 hours after in vivo MP treatment of patients. Briefly, 5x10^6 ALL cells were sonicated for 10 s and proteins were removed by ultrafiltration. Thiopurine nucleotides were calculated by subtracting the thiopurine ribonucleotides from thiopurine ribonucleosides after conversion with acid phosphatase. The concentration of TGN is expressed as pmol per 5x10^6 cells.

Mercaptopurine pharmacokinetic analysis. To assess if plasma MP and MP-metabolites concentrations were associated with intracellular TGN concentrations, we measured the plasma concentration of mercaptopurine, mercaptopurine riboside, thioguanine, thioguanine riboside and thiouric acid at three and six hours after the start of MP in 63 evaluable patients (MP, n=23; HDMTX+MP, n=17; LDMTX+MP, n=23) using the HPLC method we have previously reported.

TPMT phenotype and genotype. TPMT activity was measured in erythrocytes of 60 evaluable patients at diagnosis, as previously described. TPMT genotype was determined, using polymerase chain reaction (PCR) methods for the TPMT*2, *3A, *3B, and *3C alleles on somatic cell DNA, as previously described.

RNA extraction, gene expression profiling, and quantitative RT-PCR. All 82 patients for whom gene expression was determined had sufficient blasts in their bone marrow aspirates to permit RNA isolation from 5 to 10x10^6 leukemia cells. We extracted high-quality total RNA with TriReagent (MRC, Cincinnati, OH) from mononuclear cells.
from bone marrow aspirates at diagnosis. As previously described, high-quality total RNA was processed and hybridized to the HG-U95Av2 oligonucleotide microarray (Affymetrix Inc.; Santa Clara, CA; see manufacturer’s manual for details).

To further establish the validity of gene expression determined by microarray analysis, we performed quantitative real-time polymerase chain reaction (RT-PCR) in four patient samples for three genes (GRB2-associated binding protein 1, GAB1; heterogeneous nuclear ribonucleoprotein F, HNRPF; dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit, DPM2).

**Determination of TGN accumulation in leukemia cells in the presence or absence of a specific inhibitor of the SLC29A1 transporter.** We used the reverse-phase high-performance liquid chromatography method (HPLC) reported previously to determine the intracellular TGN concentration after treatment with 10 µM of MP (Sigma, St. Louis, MI) in the presence or absence of a specific SLC29A1 inhibitor, nitrobenzylmercaptopurine ribonucleoside (NBMPR) (Sigma). CEM, Nalm-6 and MOLT-4 human leukemia cells (American Type Culture Collection [ATCC], Manassas, VA) (50x10⁶ cells in 50 mL media) were incubated for 24 hours at 37°C and the effect of SLC29A1 transport inhibition was assessed by adding 100 nM NBMPR 20 min before and during drug exposure. As a negative control, we treated CEM cells with 1 nM of adenine (Sigma), which is not a substrate for SLC29A1, and measured the intracellular adenine concentration after 24 hour exposure in the presence or absence of 100 nM of NBMPR. These experiments were performed three times with three replicates.

**Statistical analysis.** Pair-wise comparisons using Wilcoxon rank sum test were used to assess differences in post-treatment TGN concentration among patients in the
three treatment arms. Holm’s method was used to adjust for multiple testing\(^{41}\). Wilcoxon rank sum test was used to compare plasma MP and MP-metabolite concentrations between patients randomized to HDMTX+MP and LDMTX+MP. Multiple regression analysis was used to assess the association between intracellular TGN concentrations and plasma MP and MP-metabolite concentrations separately in the group of patients treated with MP alone and with the combination of MTX+MP. Spearman’s rank correlation was used to assess the association between TPMT activity and TGN concentration. Default settings of Affymetrix Microarray Suite software version 5 (MAS 5.0, Affymetrix) were used to calculate gene expression values. Expression values were scaled to the target intensity of 2500 and log-transformed. From the total of 12,599 probe sets, those expressed in less then 5 percent of the 82 patients were omitted, leaving 5,013 probe sets for subsequent analyses. Analyses were performed separately in the two groups of patients treated with either MP alone or with the combination of MTX plus MP. Spearman’s rank correlation and false discovery rate (FDR) using Storey’s q-value\(^{42}\) were computed to identify genes associated with TGN concentration.

Leave-one-out cross-validation using support vector machine (SVM) based on Spearman’s rank correlation was used to assess the validity of association between gene expression profiles and TGN concentrations.

To assess whether the expression of gene probe sets associated with intracellular TGN concentration in patients treated with MP alone was also associated with MP sensitivity in the 60 human tumor cell line panel (NCI60), we correlated the expression levels of those genes with the growth inhibition values (GI\(_{50}\)) obtained after treatment with MP in these 60 cell lines. The GI\(_{50}\) values and the HG-U95Av2 gene expression data are publicly available through the NCI’s Developmental Therapeutics
Program (http://dtp.nci.nih.gov/mtargets/download.html). Spearman’s rank correlation was used to assess the association between gene expression and GI50 values.

R 1.8.0 statistical software was used to perform the above analyses. Principal component analysis (PCA) and 2D-hierarchical clustering were performed using GeneMaths 2.1 software (AppliedMaths, St. Martens-Latem, Belgium).
Results

*Differences in TGN accumulation in ALL cells of patients randomized to three initial treatments.* As shown in Figure 1, 33 patients who received MP alone (MP, median ±SD: 2.46±5.36 pmol/5x10^6 cells) accumulated significantly higher concentrations of TGN in their ALL cells when compared to 29 patients treated with either low-dose MTX plus MP (LDMTX+MP, median ±SD: 0.51±0.64 pmol/5x10^6 cells; p<0.001) or the 20 patients treated with high-dose MTX plus MP (HDMTX+MP, median ±SD: 0.57±0.54 pmol/5x10^6 cells; p<0.001). There was no difference in TGN concentrations between patients randomized to receive LDMTX+MP or HDMTX+MP (p=0.30). Patient characteristics (i.e., age, sex, race, WBC count and ALL subtype) were similar among the three groups of patients (Supplemental Figure B and Table A).

Therefore, based on the result of our current analysis and our previous findings that show no difference in TGN accumulation when MP is given after low-dose versus high-dose MTX\(^2\), in subsequent analyses we combined patients treated with either combination of HDMTX+MP and LDMTX+MP (MTX+MP, n=49) into one group. Multiple regression analyses did not show any association among TGN concentrations and patient’s age, sex, initial WBC count, or ALL subtype (Supplemental Table B) in either group of patients.
Figure 1: Intracellular concentration of thioguanine nucleotides (TGN) in patients after treatment with MP alone or in combination with MTX.

Box plot of TGN concentrations in ALL cells are shown for patients randomized to treatment with mercaptopurine alone (MP, n=33), mercaptopurine plus high-dose methotrexate (HDMTX+MP, n=20) or mercaptopurine plus low-dose methotrexate (LDMTX+MP, n=29). The horizontal line indicates the median for each group; the box depicts the 25th and 75th percentile range. The outlier values are shown as diamond symbols. The p-values were determined by pair-wise comparison using Wilcoxon rank sum test adjusted for multiple testing.
Correlation between mercaptopurine pharmacokinetic parameters and intracellular TGN accumulation. Plasma concentrations of MP and MP-metabolites at three and six hours after MP treatment were similar between patients randomized to treatment with HDMTX+MP or LDMTX+MP. Plasma concentrations of thioguanine and thioguanine riboside were negligible (data not shown). Multiple regression analyses did not show any significant association between plasma concentration of mercaptopurine, mercaptopurine riboside, or thiouric acid and intracellular TGN accumulation in ALL cells following treatment with MP alone (p>0.29, n=23) or with the combination of MTX+MP (p>0.20, n=40) (Supplemental Table C). The power to detect a correlation of R² equal to 0.20 at the level of alpha=0.10, using 23 and 40 individuals are 68.7% and 90%, respectively.

Association between TPMT phenotype/genotype and TGN accumulation in bone marrow ALL cells. In patients treated with MP alone (n=24) or in patients treated with the combination of MTX+MP (n=36), intracellular TGN concentrations were not associated with TPMT activity measured at the time of diagnosis (MP, R²=0.01; MTX+MP, R²=0.02). However, there were only two TPMT heterozygotes and no TPMT deficient patients among the 60 patients genotyped.

Relation between gene expression and intracellular TGN accumulation. Unsupervised hierarchical clustering, which groups patients based on predominant similarities in overall gene expression, did not cluster patients according to ALL TGN concentrations (Supplemental Figure C). However, Spearman’s rank correlation and leave-one-out cross-validation using support vector machine (SVM) identified 60 gene probe sets (rho=0.60, p<0.001, FDR=45%) (Supplemental Table D), that were associated with TGN concentrations in ALL cells following treatment with MP alone.
(n=33). Hierarchical clustering using these selected gene probe sets distinctly separated the 33 ALL patients treated with MP alone into two major groups with either low or high TGN concentration (<2.46 and >2.46 pmol/5x10^6 cells) (Figure 2A). The principal component analysis (PCA) illustrates the degree of discrimination between the two groups of patients (Figure 2B).

A
Figure 2: Hierarchical clustering using genes associated with intracellular concentration of TGN in leukemia cells of patients treated with MP alone.

(A) Hierarchical clustering of 33 ALL samples using 60 gene probe sets significantly associated with TGN accumulation in ALL cells following treatment with MP alone. Each row represents a probe set and each column a patient. Standardized expression values are shown according to the scale shown. Red and green symbols indicate patients with TGN concentrations higher and lower than 2.46 pmol/5x10^6 cells, respectively. Listed are the Affymetrix probe set IDs, gene names, Gene bank accession numbers and coefficients of correlation (rho). A positive rho indicates a positive correlation and a negative rho indicates negative correlation between gene expression and intracellular TGN concentration.

(B) Principal component analysis of ALL cells with high (red) and low
(green) TGN concentration based on the selected probe sets. Similarities are visualized in three-dimensional space.

In a separate analysis, 75 gene probe sets (\(\rho=0.65\), \(p<0.001\), FDR=25%) (Supplemental Table E) were significantly associated with TGN concentrations in ALL cells following treatment with the combination of MTX plus MP (MTX+MP, \(n=49\)). Two major branches were also found for patients treated with the combination of MTX and MP (<0.55 and >0.55 pmol/5x10^6 cells, Figure 3A); and the degree of discrimination between these two groups of patients is evident by PCA (Figure 3B).
Figure 3: Hierarchical clustering using genes associated with intracellular concentration of TGN in ALL cells of patients treated with the combination of MP and MTX.

(A) Hierarchical clustering of 49 ALL samples using 75 probe sets significantly associated with TGN accumulation in ALL cells following treatment with MTX+MP. Each row represents a probe set and each column a patient. Standardized expression values are shown according to the scale shown. Red symbols depict patients with TGN concentrations >0.55 and green symbols depict those with <0.55 pmol/5x10^6 cells. Listed are the Affymetrix probe set IDs, gene names, Gene bank accession numbers and coefficients of correlation (rho). A positive rho indicates a positive correlation and a negative rho indicates negative correlation between gene expression and intracellular TGN concentration. (B) Principal component analysis of ALL cells with high (red) and low (green) TGN concentration based on the selected probe sets. Similarities are visualized in three-dimensional space.
Interestingly, there was no overlap between the two sets of genes. Among genes associated with TGN accumulation in ALL cells following treatment with MP alone, 31 were positively and 29 negatively correlated with TGN concentrations. In the group of patients treated with the combination of MTX+MP, 50 genes were positively correlated and 25 negatively correlated with TGN accumulation. Genes significantly associated with TGN concentrations in patients treated with MP alone included xanthine oxidase \([XDH]\) (\(\rho=-0.51, p=0.002\)), solute carrier family 29 member 1 \([SLC29A1]\) (\(\rho=0.54, p=0.001\)), aldehyde dehydrogenase 1 family, member A2 \([ALDH1A2]\) (\(\rho=0.56, p<0.001\)), adenosine deaminase \([ADA]\) (\(\rho=0.48, p=0.004\)), and genes related to cell proliferation and apoptosis (i.e., caspase 7 \([CASP7]\); DNA topoisomerase II-binding protein \([TOPBP1]\); anaphase-promoting complex subunit 5 \([ANAPC5]\); and chaperonin containing TCP1, subunit 4 \([CCT4]\)). In the group of patients treated with MTX+MP, we found genes involved in ATP synthesis (i.e., solute carrier family 25, mitochondrial carrier, member 3 \([SLC25A3]\), ATP synthetase, H\(^+\) transporting, mitochondrial complex, O subunit \([ATP5O]\), cytochrome c oxidase subunit Vb \([COX5B]\) and VIIa polypeptide 2 like \([COX7A2L]\)) and several genes implicated in protein synthesis, such as ribosomal proteins (i.e., \(RPS19, RPL18, RPS25\) and \(RPL23\)) and translation factors (i.e., \(EEF1G, EIF3S5\) and \(eIF3k\)).

**Correlation between gene expression and GI\(_{50}\) using NCI’s 60 cancer cell lines.**

Using Spearman’s rank correlation, we found that among the 60 gene probe sets associated with TGN accumulation in ALL cells following in vivo treatment with MP alone, ten genes were significantly associated with the 50% growth inhibitory concentration (GI\(_{50}\)) of MP in vitro sensitivity (\(p<0.05\)) measured in a panel of 60 human tumor cell lines. Nine genes were negatively and one positively correlated with GI\(_{50}\). Six of the negatively correlated genes were positively correlated with TGN accumulation in
ALL cells in our patients. These genes encode for transporters (SLC29A1 and ATP2A3), proteins involved in oxidative phosphorylation (NADH dehydrogenase flavoprotein 1 [NDUFV1]), carbohydrate metabolism (isocitrate dehydrogenase 3 beta [IDH3B] and DNA replication [TOPBP1]) (Table 1 and Supplemental Figure D).

Table 1: Genes significantly associated with GI50 for MP in NCI’s 60 human tumor cell lines

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*highlighted in gray are genes with negative correlation with NCI60 GI50 and positive correlation with TGN concentration

TGN accumulation in leukemia cells differed after in vitro treatment with MP alone or in the presence of NBMPR. NBMPR, is an inhibitor of the SLC29A1 nucleoside transporter. As depicted in Figure 4, CEM human leukemia cells treated for 24 hour with MP in the presence of NBMPR accumulated 45% lower TGN concentrations compared to cells treated with MP only (mean±SD: 1204±262 versus 672±160 pmol/5x10⁶ cells; p<0.002), Nalm-6 cells 33% lower (mean±SD: 1133±146 versus 764±153 pmol/5x10⁶ cells; p=0.001) and in MOLT-4 cells 42% lower (mean±SD: 267±44 versus 156±62 pmol/5x10⁶ cells, p=0.006). The intracellular accumulation of adenine, which is not a substrate of SLC29A1, was not different in CEM cells treated with adenine neither in the presence nor in the absence of NBMPR (mean±SD: 47.44±8.6 versus 44.6±12.6 pmol/5x10⁶ cells, p=0.65).
Figure 4: Effect of concomitant treatment with nucleoside transporter inhibitor NBMPR on TGN and adenine concentrations in ALL cells.

Shown are intracellular TGN concentrations in CEM, Nalm-6 and MOLT-4 cells after treatment with mercaptopurine in the presence or absence of NBMPR, a nucleoside transport (SLC29A1) inhibitor nitrobenzylmercaptopurine ribonucleoside (left panel). Shown is intracellular adenine concentration in CEM cells after incubation with adenine, in the presence or absence of NBMPR (right panel). Columns represent the average of three independent experiments each with three replicates.

Validation of gene expression. Validation of gene expression levels determined by microarray analysis was performed using quantitative real-time polymerase chain reaction (RT-PCR) in four patient samples for three genes (GRB2-associated binding
protein 1, **GAB1**; heterogeneous nuclear ribonucleoprotein F, **HNRPF**; dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit, **DPM2**). The R-squared ($R^2$) between mRNA levels determined by RT-PCR and microarray was 0.97 for **GAB1**, 0.77 for **HNRPF**, and 0.83 for **DPM2**, confirming expression levels determined by the gene expression array (Supplemental Figure M1).
Discussion

Intracellular accumulation of thioguanine nucleotides (TGN) is a critical event for the cytotoxic effects of mercaptopurine\textsuperscript{8,9}. Previous studies have shown that several factors, including the activity of enzymes involved in MP metabolism, MP dose intensity, drug-drug interactions and patient compliance significantly influence inter-patient differences in TGN concentrations\textsuperscript{18,44,45}. Furthermore, these inter-patient differences have been associated with treatment efficacy\textsuperscript{3,5,24} and MP toxicity\textsuperscript{23,46}.

Well defined sources of variability include inherited differences in TPMT\textsuperscript{36,46-48} and inhibition of xanthine oxidase by allopurinol\textsuperscript{49}. Consistent with previous reports from our lab and others\textsuperscript{26,28}, the present study revealed a significant in vivo effect of MTX on intracellular accumulation of TGN, when compared to treatment with MP given alone (p<0.001). The current study also documented substantial inter-patient variability in TGN accumulation that is not explained by these drug interactions or by inherited differences in TPMT activity.

We evaluated MP with or without MTX as initial therapy, because that is the only time during which it would be possible to assess MP dispositions and effects in primary cells after in vivo treatment (i.e., by week 4 most patients have no ALL cells in their peripheral blood or bone marrow). In our study, we were focused on treatment naïve ALL and its ability to accumulate active MP metabolites in vivo. This necessitated that we assess gene expression at diagnosis and TGN accumulation after in vivo MP-treatment during the first 4 days of treatment.

In order to better understand the biological basis underlying inter-patient variability in TGN accumulation in ALL cells after MP treatment, we used oligonucleotide microarrays to perform a genome-wide determination of pre-treatment gene expression in ALL cells to identify genes and gene expression patterns that are significantly associated with high or low TGN accumulation in leukemia cells. This revealed 60 gene
probe sets (49 genes, 11 cDNA clones) that are highly related to the extent of TGN accumulation in leukemia cells of patients treated with MP alone and 75 gene probe sets (68 genes, 7 cDNA clones) of patients treated with the combination of MTX+MP. Notably, there was no overlap between the two sets of genes, indicating that different genes influence the accumulation of TGN when MP is given alone versus in combination with MTX. Previous studies have shown that in vivo gene expression changes are treatment-specific in ALL cells and that the nature of these cellular drug responses are different when medications are given alone versus in combination with a second agent\textsuperscript{37,50}. Of note, gene expression was not biased by low-dose and high-dose MTX plus MP shown by “unsupervised” clustering analysis using all genes and also not by using genes related to TGN accumulation (Supplemental Figure C, P=0.5).

Following treatment with MP alone, genes significantly related to TGN accumulation in ALL cells include genes encoding transporters (e.g. \textit{SLC29A1}, \textit{ATP2A3} and \textit{NDUFS1}), enzymes involved in purine metabolism (e.g., \textit{ADA} and \textit{XDH}), regulation of cell cycle (e.g., \textit{ANAPC5} and \textit{CCT4}) and apoptosis (e.g., \textit{CASP7} and \textit{PRKR}). Interestingly, only two (\textit{XDH} and \textit{ADA}) of the genes identified in the current study have been previously associated with MP metabolism and disposition. Expression of \textit{SLC29A1} (or \textit{ENT1}), a member of the family of the equilibrative nucleoside transporters which mediates uptake of nucleosides and chemotherapeutic agents\textsuperscript{39,51}, has been shown to be positively associated with cytotoxicity of nucleoside analogues (e.g., MP, azacytidine, cytarabine and inosine-glycodialdehyde) in human cancer cell lines\textsuperscript{52} and clinical response to cytarabine in patients with acute myeloid leukemia\textsuperscript{53}. Consistent with this, in our patients, \textit{SLC29A1} expression was high in ALL cells that accumulated high post-treatment TGN concentrations (rho=0.54, p=0.0015).

We measured TGN accumulation in CEM, Nalm-6, MOLT-4 human leukemia cells after exposure to MP in the presence or absence of NBMPR, a specific inhibitor of
the SLC29A1 transporter. Inhibition of SLC29A1 by NBMPR reduced the concentration of TGN at 24 hours by 45%, 33%, and 42% respectively (p<0.006). Together, these in vivo and in vitro data provide the first evidence that inhibition of SLC29A1 reduced steady-state TGN accumulation by 45-33% and this also indicates the existence of other MP and TGN transport and metabolic mechanisms in ALL cells.

**XDH** encodes xanthine oxidase, an important enzyme involved in the first-pass metabolism of MP\textsuperscript{16}. This enzyme, predominately expressed in the intestinal mucosa and liver, converts MP into inactive thiouric acid\textsuperscript{54,55}, a principal pathway for MP catabolism. Although **XDH** mRNA was detectable in the majority of ALL samples analyzed (median of expression =1575), it was expressed at a relatively low level. The significant negative correlation between XDH expression and TGN concentration in ALL cells (rho=-0.52, p=0.0028) is consistent with the role of this enzyme in the catabolism of MP, but may be related to concordant variability of XDH in other tissues (e.g., liver). The non-significant correlation of XDH expression and TGN (rho=0.08, p=0.54) in patients treated with MTX+MP, may be due to MTX's inhibitory effects on xanthine oxidase activity\textsuperscript{25}.

Another gene that showed a positive relation between gene expression and TGN accumulation (rho=0.48, p=0.0046) was human adenosine deaminase (**ADA**). **ADA** encodes a purine-metabolizing enzyme that indirectly influences the metabolism of MP through perturbation of the intracellular pool of enzymes involved in nucleotide synthesis. ADA has been extensively studied in ALL patients and low ADA activity has been associated with a poor outcome after in vivo treatment with the structurally similar 6-thioguanine\textsuperscript{56}. It is postulated that high ADA expression results in accumulation of inosine and subsequently inosine monophosphate (IMP)\textsuperscript{57}, which inhibits phosphoribosyl pyrophosphate amidotransferase (PPAT), thereby increasing intracellular phosphoribosyl pyrophosphate (PRPP), which is a rate-limiting precursor for the formation of TGN\textsuperscript{58}. 
Interestingly, among the 60 gene probe sets significantly associated with TGN accumulation in patients treated with MP alone, ten were significantly correlated with MP sensitivity (GI50) in NCI’s 60 human tissue cell lines. Notably, six of these genes were negatively correlated with GI50 in cell lines and positively with TGN accumulation in our patients. These findings indicate that increased expression of genes associated with TGN accumulation is associated with increased sensitivity to MP.

Among genes related to TGN accumulation after MTX+MP treatment, were several genes involved in mitochondrial activities (e.g., ATP biosynthesis and fatty acid metabolism) and protein synthesis (e.g., ribosomal proteins and translation factors). To our knowledge, none of those 68 genes has been previously implicated in MP metabolism, TGN accumulation or thiopurine resistance. Four of these genes, SLC25A3, ATP50, COX5B and COX7A2L, are involved in the synthesis of the structurally similar nucleotide, ATP. SLC25A3 encodes the phosphate carrier (PCH) that is an integral protein of the mitochondrial inner membrane and is involved in the terminal process of oxidative phosphorylation59. ATP50 encodes for oligomycin sensitivity conferring protein (OSCP) subunit of the stalk of mitochondrial respiratory chain F1F0-ATP synthase60. Finally, COX5B and COX7A2L encode for subunits of the cytochrome c oxidase, the terminal enzyme of the mitochondrial electron transport chain. ATP is a cofactor for GMPS61, an important enzyme involved in conversion of thioxanthosine monophosphate (TXMP) to thioguanine monophosphate (TGMP), an essential step in TGN formation. Low ATP levels have been associated with significantly lower intracellular TGN accumulation in leukemia cells treated in vitro with MP62. MTX, through the inhibition of de novo purine synthesis, decreases intracellular levels of ATP44;63. Therefore, it is plausible that patients with increased expression of SLC25A3, ATP50, COX5B and COX7A2L have higher ATP levels and are therefore less susceptible to inhibition of ATP synthesis by MTX and as a consequence achieve relatively higher intracellular TGN
concentrations when compared to other patients treated with the MTX+MP combination. Expression of these genes was not significantly associated with TGN concentrations in the group of patients treated with MP alone.

Together, these in vivo and in vitro data provide new insights into the genomic basis of inter-patient differences in TGN accumulation in ALL cells, and reveal significant differences when MP is given alone or in combination with MTX.

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SUPPLEMENTAL MATERIAL IS AVAILABLE ONLINE AT THE TIME OF FINAL PUBLICATION ONLY.
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Gene expression and thioguanine nucleotide disposition in acute lymphoblastic leukemia after in vivo mercaptopurine treatment

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