

Differing Contribution of Thiopurine Methyltransferase to Mercaptopurine *versus* Thioguanine Effects in Human Leukemic Cells¹

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ABSTRACT

Thioguanine and mercaptopurine are prodrugs requiring conversion into thiopurine nucleotides to exert cytotoxicity. Thiopurine S-methyltransferase (TPMT), an enzyme subject to genetic polymorphism, catabolizes thiopurines into inactive methylated bases, but also produces methylthioguanine nucleotides and methylmercaptopurine nucleotides from thioguanine and mercaptopurine nucleotides, respectively. To study the effect of TPMT on activation *versus* inactivation of mercaptopurine and thioguanine, we used a retroviral gene transfer technique to develop human CCRF-CEM cell lines that did (TPMT+) and did not (MOCK) overexpress TPMT. After transduction, TPMT activities were 14-fold higher in the TPMT+ *versus* the MOCK cell lines ($P < 0.001$). TPMT+ cells were less sensitive to thioguanine than MOCK cells ($IC_{50} = 1.10 \pm 0.12 \mu\text{M}$ *versus* $0.55 \pm 0.19 \mu\text{M}$; $P = 0.02$); in contrast, TPMT+ cells were more sensitive to mercaptopurine than MOCK cells ($IC_{50} = 0.52 \pm 0.20 \mu\text{M}$ *versus* $1.50 \pm 0.23 \mu\text{M}$; $P < 0.01$). The lower sensitivity of TPMT+ *versus* MOCK cells to thioguanine was associated with lower thioguanine nucleotide concentrations (917 ± 282 *versus* 1515 ± 183 pmol/ 5×10^6 cells; $P = 0.01$), higher methylthioguanine nucleotide concentrations (252 ± 34 *versus* 27 ± 10 pmol/ 5×10^6 cells; $P = 0.01$), less inhibition of *de novo* purine synthesis (13 *versus* 95%; $P < 0.01$), and lower deoxythioguanosine incorporation into DNA ($2.0 \pm 0.6\%$ *versus* $7.2 \pm 2.0\%$; $P < 0.001$). The higher sensitivity of TPMT+ cells to mercaptopurine was associated with higher concentrations of methylmercaptopurine nucleotide (2601 ± 1055 *versus* 174 ± 77 pmol/ 5×10^6 cells; $P = 0.01$) and greater inhibition of *de novo* purine synthesis (>99% *versus* 74%; $P < 0.01$) compared with MOCK cells. We conclude that methylation of mercaptopurine contributes to the antiproliferative properties of the drug, probably through inhibition of *de novo* purine synthesis by methylmercaptopurine nucleotides, whereas thioguanine is inactivated primarily by TPMT.

INTRODUCTION

Mercaptopurine and thioguanine are thiopurine drugs currently administered in the treatment of acute lymphoblastic and myeloblastic leukemia. Their precise mechanism of action remains unclear despite >40 years of *in vivo* and *in vitro* investigations (1). Mercaptopurine and thioguanine require intracellular conversion by HPRT³ into thiopurine nucleotides to exert cytotoxicity (2). In cells, mercaptopurine (the analogue of hypoxanthine) and thioguanine (the analogue of guanine) are converted by HPRT into TIMP and TGMP, respectively,

and subsequently to the active cytotoxic TGNs, which are incorporated into DNA in the reduced form (deoxythioguanosine; Refs. 3–5). TIMP and TGMP are methylated by TPMT (EC 2.1.1.67), an S-adenosylmethionine-dependent cytosolic enzyme, into MeTIMP and MeTGMP, respectively (Fig. 1; Refs. 6, 7).

TPMT is subject to a common genetic polymorphism, with ~10% of individuals heterozygous and 0.3% homozygous mutant at the *TPMT* locus (8). Patients with low TPMT activity have greater hematological toxicity after standard doses of mercaptopurine, thioguanine, or azathioprine than do patients with wild-type *TPMT* (9–12). Such patients have very high concentrations of active TGNs, with low or absent methylthiopurine metabolite concentrations. However, the potential contributions of MeTGMP and MeTIMP to the pharmacological properties of thioguanine and mercaptopurine, respectively, remain unclear. Methylation of mercaptopurine and thioguanine bases is an inactivating pathway, shunting parent drugs away from the HPRT activation pathway. However, methylation of TIMP or administration of methylmercaptopurine riboside results in inhibition of DNPS (13, 14). *In vivo* observations show that patients with low TPMT activity tolerate higher concentrations of TGNs than do patients with wild-type *TPMT* (9, 10, 15). In addition, incorporation of deoxythioguanosine into DNA of various human B-lineage and T-lineage cell lines does not correlate with their sensitivity to mercaptopurine (5). Thus, these *in vivo* and *in vitro* data suggest that methylation could contribute to the effects of thiopurines.

To clarify whether and by what mechanism TPMT contributes to cytotoxicity, we have developed isogenic human leukemic cell lines expressing recombinant human TPMT and compared the cytotoxic effects of mercaptopurine and thioguanine.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Human kidney 293T cells, murine fibroblast NIH-3T3 cells, and human HeLa cells were maintained in DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) and 2 mM glutamine. Human CCRF-CEM cells (human acute lymphoblastic leukemia, T lineage) were purchased from the American Type Culture Collection and were maintained in RPMI 1640 (BioWhittaker) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine. All cells were maintained in log phase at 37°C in a 5% CO₂ humidified atmosphere.

Preparation of Replicative Incompetent Retroviruses. The functional human *TPMT* cDNA (16) was subcloned into the MSCV-IRES-GFP vector at the *EcoRI* and *NsiI* sites upstream of an IRES, driving the expression of GFP (17). All plasmids were purified by standard cesium chloride gradient centrifugation. Retroviruses were prepared by transient cotransfection of the vector and helper plasmids in the packaging cell line 293T cells (18). Murine amphotropic retroviruses were prepared by cotransfection of 15 μg of MSCV-IRES-GFP (MOCK vector) or MSCV-TPMT-IRES-GFP (TPMT+ vector) and 15 μg of the helper plasmid PEQ-PAM3 (encoding the envelope, nucleocapsid, and reverse transcriptase) by standard calcium phosphate precipitation (19). RD114-pseudotyped retroviruses containing the feline endogenous virus envelope (20, 21) were prepared by cotransfection of 10 μg of MSCV-IRES-GFP or MSCV-TPMT-IRES-GFP vector with 10 μg of helpers pRDF (encoding the envelope) and pEQ-PAM3-E (encoding the nucleocapsid and reverse transcriptase), using Fugene-6 transfection reagent (Roche Diagnostics, Indianapolis, IN). Twenty-four h after transfection, retroviral supernatants were

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³ The abbreviations used are: HPRT, hypoxanthine phosphoribosyltransferase; TIMP, thioinosine monophosphate; TGMP, thioguanosine monophosphate; TGN, thioguanine nucleotide; TPMT, thiopurine methyltransferase; MeTIMP, methylthioinosine monophosphate; MeTGMP, methylthioguanine monophosphate; DNPS, *de novo* purine synthesis; IRES, internal ribosomal entry site; GFP, green fluorescence protein; FACS, fluorescence-activated cell sorting; TIN, 6-thioinosine nucleotide; MeMPN, methylmercaptopurine nucleotide; MeTGN, methyl 6-thioguanine nucleotide; MeTG, methyl thioguanine.

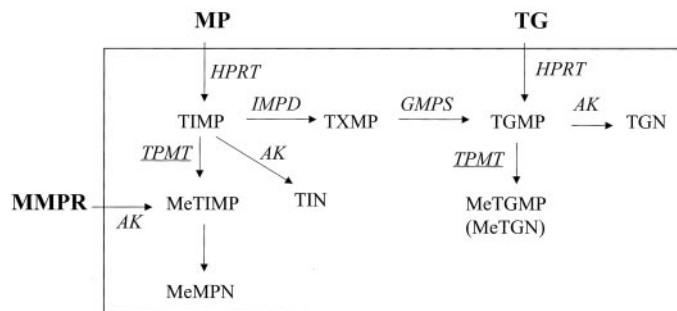


Fig. 1. Intracellular metabolism of mercaptopurine (MP), thioguanine (TG), and methylmercaptopurine riboside (MMPR). Abbreviations: TXMP, thioxanthosine monophosphate; IMPD, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase; AK, adenosine kinase. Note: MeTGMP is known to exist, but it is not clear whether methylthioguanosine di- or triphosphate is present. Our assay did not distinguish between MeTGMP and other MeTGNs. Not shown is inactivation of mercaptopurine and thioguanine by TPMT to their respective methylated bases and by xanthine oxidase in the gastrointestinal tract and liver.

harvested every 6–12 h for up to 72 h. Retroviral supernatants were pooled, filtered, and stored at -70°C .

Titers (number of viral particles per ml of supernatant) were estimated using a 1:10 dilution of the retroviral supernatant. The functional integrity of the RD114 retroviruses was assessed in HeLa cells prior to transduction in CEM.

Retroviral Transduction of Cells. Murine NIH-3T3 cells (2.0×10^5 cells/10-cm dish) were transduced with amphotropic retroviral supernatants in the presence of 10 $\mu\text{g}/\text{ml}$ Polybrene (Sigma Chemical Co., St. Louis, MO). GFP expression was scored by FACS 72 h after transduction. The CEM cells were transduced using the RD114 retroviruses in the presence of Polybrene (final concentration, 8 $\mu\text{g}/\text{ml}$) and retronectin (recombinant human fibronectin fragment CH-296; TAKARA, Otsu, Japan). Retronectin was coated on 6-well Costar plates (Corning Inc., New York, NY) at a concentration of 20 $\mu\text{g}/\text{cm}^2$, and 4 ml of retroviral supernatant were preloaded on the retronectin over 30 min at 37°C and then discarded. Subsequently, a total of 10^6 cells resuspended in 5 ml of retroviral supernatant were loaded on the plates. This transduction procedure was repeated twice over 48 h. CEM cells expressing GFP were sorted by FACS 1 week after transduction. Control retroviruses (MOCK) or retroviruses containing the TPMT insert (TPMT+) were prepared concomitantly. Recombinant MOCK versus TPMT+ cells were studied at comparable passage numbers after transduction. Every 1–2 weeks, GFP levels were determined by FACS analysis together with TPMT activity.

Enzymatic Assays: HPRT and TPMT Activity. TPMT activity in cell lysates was determined by the nonchelated radiochemical assay of Weinschilboum *et al.* (22) as reported previously by our laboratory (23) with the exception that the cofactor S -[^{14}C]adenosylmethionine was used at 50 μM . One unit of enzymatic activity corresponds to the formation of 1 nmol of methylmercaptopurine per hour of incubation. Results are normalized to 1×10^9 cells (units/ 10^9 cells). HPRT activity in cell lysates was estimated by the formation of [^{14}C]inosine monophosphate from [^{14}C]hypoxanthine as reported previously. Results are expressed as nmol/h/ 10^6 cells (5).

Cytotoxicity Assays. Mercaptopurine, thioguanine, methylmercaptopurine riboside, and methotrexate were all purchased from Sigma. Mercaptopurine was dissolved in culture media and filtered (0.45 μm filters); the concentration was calculated using the UV molar extinction coefficient ($\epsilon_{340} = 19600$). Methylmercaptopurine riboside, thioguanine, and methotrexate were dissolved in DMSO (final concentration always $<0.1\%$ in the culture media). MOCK cells (corresponding to GFP-expressing cells transduced with the retrovirus control) and TPMT+ cells (corresponding to GFP-expressing cells transduced with the retrovirus containing TPMT insert) in log phase were treated with drugs and compared with cells treated with solvent alone. Cell viability was assessed using annexin staining and FACS analysis. Cytotoxicity was expressed as the ratio of viable cells incubated with drugs (i.e., cell count times the percentage of viable cells) to the number of viable cells (cell count times the percentage of viable cells) in the untreated controls. The proliferation and dose-response curve in the presence or absence of drugs were assessed using the CellTiter 96 AQ_{ueous} Assay (Promega, Madison, WI) in 96-well plates as recommended by the manufacturer.

Determination of Cytosolic Thiopurine Nucleotide Concentrations. We used modifications of the reverse-phase high-performance liquid chromatography method reported previously (7). Cells were centrifuged 5 min at 1500 rpm and washed with two volumes of PBS; pellets were resuspended in 200 μl of saline and stored at -70°C . After thawing, 200 μl of Tris-EDTA buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8)] were added. Cell suspensions were sonicated (20 s) and filtered using Centricon-3K filters (Millipore, Bedford, MA). The cell extract was treated with bacterial alkaline phosphatase (Promega) for 2 h at 56°C (final concentration, 26 units/ml) to convert the thiopurine ribonucleotides (mono-, di-, or triphosphates) into their respective ribonucleosides. TINs (6-thioinosine mono-, di-, or triphosphates) were converted into 6-thioinosine (i.e., mercaptopurine riboside); TGNs (6-thioguanosine mono-, di-, or triphosphates) into 6-thioguanine riboside; and MeMPNs (methyl 6-thioinosine mono-, di-, or triphosphates) into 6-methylmercaptopurine riboside. MeTGN concentrations were determined using an acid hydrolysis procedure (24), in which any methylated thioguanosine mono-, di-, or triphosphate would be converted to MeTG. A 300- μl cell extract was heated for 1 h at 100°C in the presence of 15 μl of perchloric acid (20%) to allow the conversion of MeTGN to MeTG. Concentrations were determined by comparison with a standard curve of MeTG treated in the same conditions.

DNPS Assay. The rate of DNPS was assessed as the pmol of radiolabeled purine bases (adenine and guanine) relative to nonlabeled purines (expressed in nmol) after a 2-h *in vitro* incubation with [^{14}C]formate, as described previously (25).

Incorporation of Deoxythioguanosine into Genomic DNA. Genomic DNA was isolated from 10×10^7 cells after incubation of cells with thioguanine or mercaptopurine, using the Qiagen cell culture DNA kit (Qiagen, Chatsworth, CA), according to the manufacturer's instructions. Quantitation of deoxythioguanosine in genomic DNA was monitored by high-performance liquid chromatography after enzymatic hydrolysis of 10 μg of DNA as reported previously (5).

Flow Cytometry Analysis and Cell Viability Assessment. GFP-expressing cells were selected by flow cytometry with a FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA) using excitation at 488 nm and fluorescence detection at 530 nm. Propidium iodide (Sigma) was added to all samples before analysis to allow identification and elimination of dead cells from the analysis. Cell viability was determined by annexin staining. Approximately 5×10^5 cells were washed in PBS and resuspended in 100 μl of staining buffer (10 mM HEPES, 0.9% NaCl, 2.5 mM CaCl_2 , 0.1% BSA) containing 10 μl of phycoerythrin-conjugated Annexin-V (Annexin-V-PE; PharMingen, San Diego, CA) and incubated at room temperature in the dark for 15 min, followed by the addition of 500 μl of staining buffer containing 2.5 μg of propidium iodide. The samples were filtered through 40 μm nylon mesh and analyzed on a Becton Dickinson FACS Calibur flow cytometer, collecting green fluorescence from GFP, orange fluorescence from Annexin-V-PE, and red fluorescence from propidium iodide.

Statistical Analysis. All statistics were performed using nonparametric tests and Statistica Software (Statsoft, Tulsa, OK). All results were expressed as mean \pm SD from three separate experiments with three replicates, or as otherwise indicated. The Mann-Whitney *U* test was used for between-group comparisons, and the Spearman rank test (*R*) was used for correlations between parameters. Concentrations producing 50% inhibition of the proliferation (IC_{50}) were determined using the WinNonlin software (PharSight, Mountain View, CA).

RESULTS

Retroviral Gene Transfer and Expression of Human TPMT. Retroviral titers for the MOCK and the TPMT+ amphotropic retroviruses were $0.9 \times 10^6/\text{ml}$ and $1.0 \times 10^6/\text{ml}$, respectively. Amphotropic retroviruses were transduced in the NIH-3T3 cells with an efficiency ranging from 96 to 100% (percentage of cells gated GFP positive by comparison with a negative control of untransduced cells). Transduction of the TPMT-containing retrovirus in 3T3 cells yielded a 22-fold increase of TPMT activity relative to MOCK cells ($P < 0.001$), whereas transduction of the retrovirus control (MOCK) had no effects on TPMT activity ($P = 0.40$ compared with untransduced cells; Fig. 2).

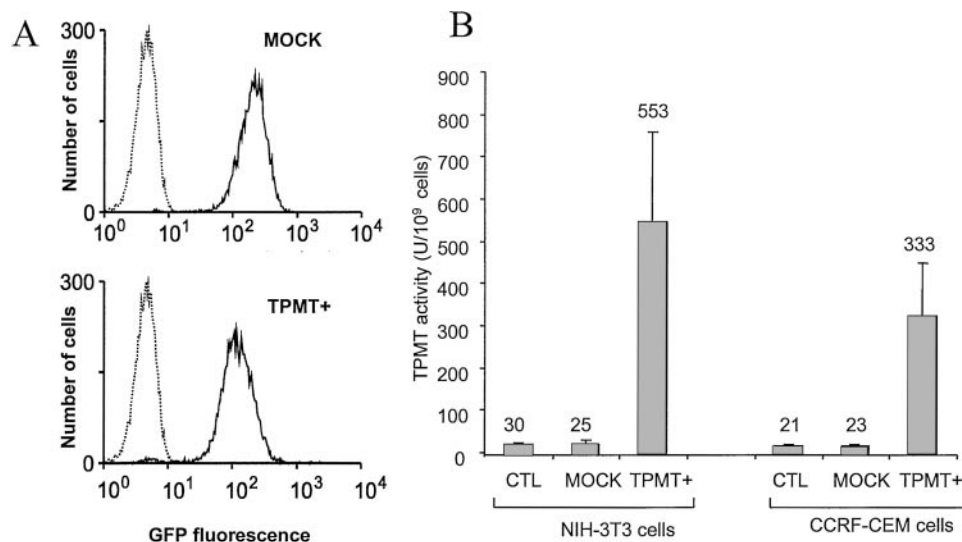


Fig. 2. Retroviral gene transfer of TPMT. *A*, 1 week after cell sorting for GFP positivity, CEM-MOCK (top) and CEM-TPMT+ (bottom) expressed similar levels of GFP. Dotted lines, negative control (untransduced cells); solid lines, GFP levels in the transduced cells. 3T3-GFP-expressing cells gave a similar pattern of GFP expression, but no sorting was required (transduction efficiency >96%). *B*, TPMT activity in 3T3 and CEM cells untransduced (CTL) or transduced with the retrovirus control (MOCK) or retrovirus containing the TPMT insert (TPMT+). All TPMT activities were determined during the 2 months following transduction. Columns represent average TPMT activities from at least seven independent assays determined every 1–2 weeks after transduction, using two separate batches of cells transduced with the retroviruses; bars, SD.

The efficiency of transduction with amphotropic retroviruses was lower with human CCRF-CEM human T lymphoblasts (only 10% of GFP-positive cells for TPMT retrovirus) than with 3T3 cells. To achieve a higher efficiency of transduction, we used a combination of retronectin and RD114-pseudotyped retroviral particles, which allowed adequate recoveries of GFP-positive cells (85 and 40% for the MOCK and TPMT+ retroviruses, respectively; average of two separate experiments). After flow cytometry sorting, CEM-TPMT+ cells had 14-fold higher TPMT activity than did the CEM-MOCK cells (Fig. 2).

Effects of Thioguanine, Mercaptopurine, and Methylmercaptopurine Riboside. Prior to cytotoxicity studies, we determined whether transduction with TPMT could have modified critical parameters of thiopurine responsiveness. CEM-TPMT+ cells were comparable to the CEM-MOCK cells with regard to the doubling time (24.4 ± 1.1 versus 24.5 ± 2.1 h; $P = 0.82$), DNPS rate (23.3 ± 7.8 versus 26.2 ± 8.5 pmol/nmol/h; $P = 0.44$), and HPRT activities (8.6 ± 5.2 versus 8.6 ± 3.9 nmol/h/10⁶ cells; $P = 1.0$). Similarly, no significant differences in these parameters were found between the 3T3-MOCK and 3T3-TPMT+ cells (not shown).

We investigated the inhibitory effects of mercaptopurine, thioguanine, and methylmercaptopurine riboside on proliferation in the CEM-MOCK and the CEM-TPMT+ cells (Table 1). As expected, after 48 h of incubation with methylmercaptopurine riboside, which bypasses the methylation by TPMT, we observed no differences in sensitivity between the MOCK and the TPMT+ cells ($P = 0.38$). In addition, methotrexate yielded no significant difference of sensitivity ($P = 0.27$) between MOCK and TPMT+ cells. However, the treatment of cells with mercaptopurine or thioguanine showed significant and opposite differences. After 48 h of incubation with thioguanine, a 2-fold lower IC₅₀ for the MOCK cells was observed compared with the TPMT+ cells ($P = 0.02$); in contrast, after mercaptopurine incubation, a 2.9-fold higher IC₅₀ was observed for the MOCK cells compared with the TPMT+ cells ($P < 0.01$). The TPMT+ cells exhibited a 2-fold lower IC₅₀ after mercaptopurine treatment compared with after thioguanine treatment ($P = 0.01$). In contrast, MOCK cells had a 2.7-fold higher IC₅₀ after mercaptopurine treatment compared with after thioguanine treatment ($P = 0.01$). A similar pattern of sensitivity was seen in the 3T3 recombinant cells: after 48 h of incubation with mercaptopurine (10 μM), we observed a greater inhibition of proliferation ($P = 0.01$) in the 3T3-TPMT+ cells (inhibition $73 \pm 14\%$ relative to control) than in the 3T3-MOCK cells (inhibition $47 \pm 10\%$ relative to control).

We determined cell viability by annexin staining after 24 or 48 h of treatment with mercaptopurine (10 μM), thioguanine (1 μM), or methylmercaptopurine riboside (1 μM) in the CEM recombinant cells. For all compounds tested, no significant cytotoxic effect was observed after 24 h of treatment (viability >90% compared with untreated controls); this is consistent with the delayed effects of thiopurines. After 48 h of incubation with mercaptopurine, lower viability was observed in the CEM-TPMT+ cells compared with CEM-MOCK cells ($P < 0.01$; Fig. 3). In contrast, after thioguanine treatment, higher viability was observed in the CEM-TPMT+ cells compared with the CEM-MOCK cells ($P < 0.01$). No significant differences in cell viability were observed after 48 h of incubation with methylmercaptopurine riboside in the MOCK cells compared with TPMT+ cells ($P = 0.54$).

Cytosolic Thiopurine Metabolites. We compared the cytosolic thiopurine nucleotide concentrations in the MOCK and TPMT+ cells after incubation with mercaptopurine (10 μM), thioguanine (1 μM), and methylmercaptopurine riboside (1 μM). After 24 h incubation with mercaptopurine, the CEM-TPMT+ cells had lower concentrations of TINs ($P = 0.007$), comparable levels of TGNs ($P = 0.8$), and 15-fold higher concentrations of MeMPNs ($P < 0.001$) than the CEM-MOCK cells (Fig. 4). The total thiopurine nucleotide concentration (TIN + TGN + MeMPN) in the CEM-MOCK cells (7926 ± 2143 pmol/5 × 10⁶ cells) was similar to that of the CEM-TPMT+ cells (7621 ± 3590 pmol/5 × 10⁶ cells; $P = 0.72$). After incubation for 48 h with mercaptopurine, TGN levels were lower in the CEM-TPMT+ cells (100 ± 92 pmol/5 × 10⁶ cells) than in the CEM-MOCK cells (298 ± 49 pmol/5 × 10⁶ cells; $P < 0.05$). Similarly, after 48 h of incubation with mercaptopurine (10 μM), 3T3-TPMT+ cells had lower concentrations of TGN compared with

Table 1 Average IC₅₀s (± SD) for thioguanine, mercaptopurine, methylmercaptopurine riboside, and methotrexate in CEM-MOCK and CEM-TPMT+ cells after 48 h of incubation

Inhibition of proliferation was determined using the CellTiter 96 AQ_{ueous} Assay (Promega) with colorimetric detection of formazan formed at 490 nm. Results summarize three to five experiments at different passage numbers, with four replicates each.

Compound	IC ₅₀ (μM)		P
	MOCK	TPMT+	
Thioguanine	0.55 ± 0.19	1.10 ± 0.12	0.02
Mercaptopurine	1.50 ± 0.23	0.52 ± 0.20	<0.01
Methylmercaptopurine riboside	0.072 ± 0.046	0.054 ± 0.029	0.38
Methotrexate	0.012 ± 0.005	0.012 ± 0.006	0.27

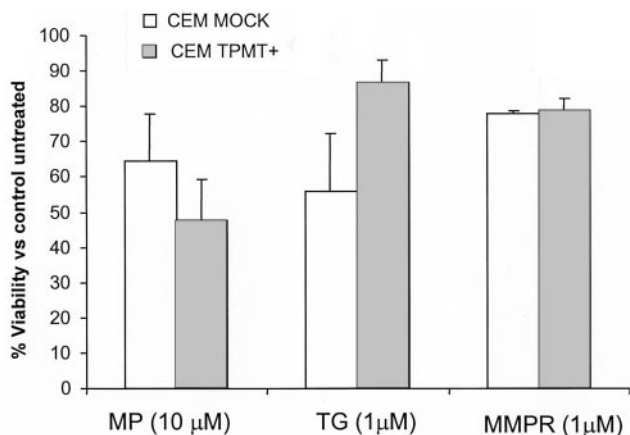


Fig. 3. Viability of CEM isogenic cell lines after incubation with 10 μM mercaptopurine (MP), 1 μM thioguanine (TG), or 1 μM methylmercaptopurine riboside (MMPR). Cells in log phase were treated for 48 h with drugs and stained with annexin as described in "Materials and Methods." Results are expressed as mean ± SD (bars). Columns represent the average of four to seven independent experiments for thioguanine and mercaptopurine and two experiments for methylmercaptopurine riboside.

3T3-MOCK cells (67 ± 46 pmol/5 × 10⁶ cells versus 124 ± 58 pmol/5 × 10⁶ cells; $P = 0.05$) and higher concentrations of MeMPN (7296 ± 4385 pmol/5 × 10⁶ cells versus 655 ± 207 pmol/5 × 10⁶ cells; $P < 0.01$; not shown).

Thioguanine, which is directly processed by HPRT into TGNs, also showed significant differences between CEM-MOCK and CEM-TPMT+ cells. After 24 h of incubation with thioguanine (1 μM), we observed significantly lower TGN concentrations in the TPMT+ compared with the MOCK cells ($P < 0.01$), and 9-fold higher concentrations of MeTGNs ($P < 0.01$) in TPMT+ cells (Fig. 4). However, the total concentration of thiopurine nucleotides after thioguanine incubation was slightly but significantly ($P = 0.02$) higher in the MOCK (1542 ± 192 pmol/5 × 10⁶ cells) compared with the TPMT+ cells (1132 ± 215 pmol/5 × 10⁶ cells). After 24 h of treatment with methylmercaptopurine riboside, comparable MeMPN levels in the MOCK and the TPMT+ cells were observed (4383 ± 475 versus 4471 ± 599 pmol/5 × 10⁶ cells; $P = 0.58$). Thus, the higher cytotoxic effect observed in the TPMT+ cells compared with MOCK cells after mercaptopurine incubation was correlated with higher MeMPN levels, whereas the higher survival of the TPMT+ cells compared with the MOCK cells after thioguanine incubation was correlated with lower TGN concentrations.

Incorporation of Deoxythioguanosine into Genomic DNA. The percentage of incorporation of deoxythioguanosine into genomic DNA, expressed as the percentage of all guanosine substituted by deoxythioguanosine after mercaptopurine (10 μM) or thioguanine (1 μM) treatment, is shown in Fig. 5. After 24 or 48 h of treatment with mercaptopurine, CEM-MOCK cells had 1.6-fold higher percentage of deoxythioguanosine incorporation into their DNA compared with CEM-TPMT+ cells ($P < 0.01$). After 24 h of treatment with thioguanine, CEM-MOCK cells had 1.7-fold higher percentage of deoxythioguanosine incorporation ($P < 0.01$), which reached a 3.6-fold higher range at 48 h ($P < 0.01$) when compared with the CEM-TPMT+ cells. Thus, after 48 h of treatment, the percentage of incorporation of deoxythioguanosine into the DNA of MOCK cells treated with thioguanine was higher than that observed after MOCK cells were treated with mercaptopurine ($P = 0.03$).

Effects on DNPS. The CEM-TPMT+ cells treated with mercaptopurine (10 μM) exhibited more rapid and pronounced inhibition of DNPS compared with CEM-MOCK cells (Fig. 6). The DNPS rate was inhibited by >95% in the CEM-TPMT+ cells after only 12 h of

treatment, whereas complete inhibition was observed in the CEM-MOCK cells only after 48 h. In contrast, CEM-TPMT+ cells treated with thioguanine (1 μM) had only 13% DNPS inhibition after 48 h of treatment, whereas the CEM-MOCK cells showed 95% inhibition ($P < 0.01$). After 48 h of treatment with mercaptopurine (10 μM), DNPS was completely inhibited in the 3T3-TPMT+ cells ($99.6 \pm 0.3\%$), whereas in the 3T3-MOCK cells, we observed a lower level of inhibition ($65.2 \pm 7.7\%$; $P = 0.02$).

The MeMPN concentration after mercaptopurine treatment in the CEM-MOCK and TPMT+ cells was highly correlated with the percentage of DNPS inhibition ($R = -0.94$; $P < 0.001$), and the concentration of MeMPNs inducing a 50% inhibition of DNPS was 224 pmol/5 × 10⁶ cells (Fig. 7). TGN concentrations did not correlate with DNPS inhibition after mercaptopurine treatment ($P > 0.90$), but in the case of thioguanine treatment, higher TGN concentrations correlated with higher DNPS inhibition in the CEM recombinant cells ($P = 0.02$; data not shown).

DISCUSSION

TPMT, a cytosolic enzyme subject to a common genetic polymorphism, is partly responsible for variations in thiopurine response among patients. Patients with low TPMT activities are at greater risk of hematological side effects during thiopurine treatment and exhibit increased intracellular accumulation of active nonmethylated TGNs (9, 10). Methylation of mercaptopurine and thioguanine bases is inactivating, leaving less parent substrate available for activation through the HPRT pathway. The mechanism of antitumor action of thiopurines is hypothetically related to the incorporation of TGNs into DNA (2, 4). This idea is supported by the fact that the inhibition of this incorporation protects cells against the delayed effect of mercaptopurine or thioguanine (4, 26). However, there are several pieces of

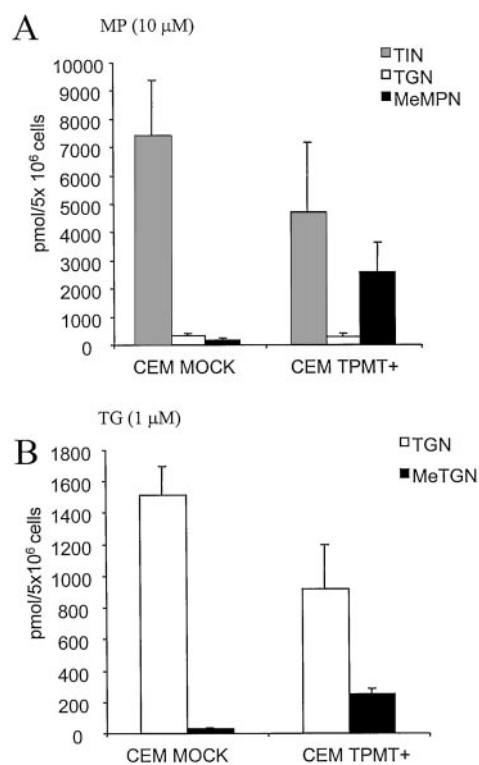
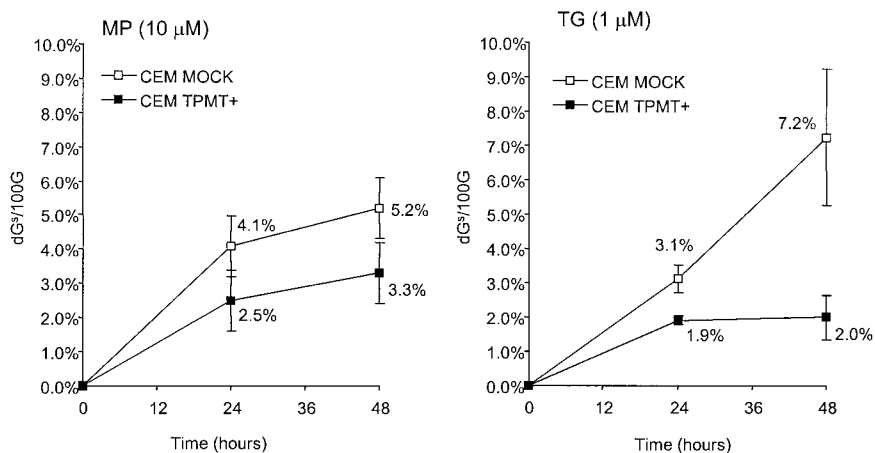


Fig. 4. Intracellular concentration of thiopurine nucleotides in CEM cells. Intracellular thiopurine nucleotide concentrations after incubation for 24 h with 10 μM mercaptopurine (A) or 1 μM thioguanine (B). Columns represent the average of three independent experiments each with three replicates; bars, SD.

Fig. 5. Deoxythioguanine (dG^*) incorporation into genomic DNA of CEM cells. CEM-MOCK and CEM-TPMT+ cells were treated with $10 \mu\text{M}$ mercaptopurine (MP; left) or $1 \mu\text{M}$ thioguanine (TG; right), and genomic DNA was extracted after 24 or 48 h of treatment. Results are the percentage of guanosine substituted with deoxythioguanosine, presented as the average \pm SD (bars) from two to three separate experiments with three or four replicates at each time point. At each time point, the TPMT+ cells had significantly lower deoxythioguanosine incorporation compared with the MOCK cells ($P < 0.01$).



evidence that suggest that TPMT may contribute to activation of thiopurines, particularly mercaptopurine. In this study, we used isogenic cell lines that express high *versus* low levels of TPMT to show that TPMT may contribute to the cytotoxic and pharmacological effects of mercaptopurine.

We chose CCRF-CEM cells because they are known to be sensitive to thiopurines and they express relatively low TPMT activity constitutively (7). The 14-fold higher TPMT activity we observed in our TPMT+ cells compared with the MOCK cells after transduction shows a similar fold range of activity (lowest to highest) as has been observed in leukemic blasts of patients with acute lymphoblastic leukemia (29-fold; Ref. 23). In addition, we found comparable effects of TPMT on thiopurine pharmacology in murine NIH-3T3 cells and in human CEM cells. We acknowledge that the absolute level of TPMT in both the MOCK and TPMT+ CCRF-CEM cells is higher than the levels measured in patient leukemic blasts (3.3–88.9 units/ 10^9 cells; Ref. 23), and thus caution should be exercised in extrapolating the results presented here directly to the clinical situation. However, the qualitative metabolite profile (ratio of methyl metabolites to TGN) that we measured after mercaptopurine and thioguanine incubation in TPMT+ cells is much closer to the ratios observed *in vivo* in patients with wild-type TPMT after mercaptopurine (9) and thioguanine (27) incubation than those observed with our MOCK cells (Fig. 4), suggesting that the results presented here have some applicability to the clinic.

Mercaptopurine is converted by HPRT into TIMP and subsequently to TGMP by two steps involving inosine monophosphate dehydrogenase and guanosine monophosphate synthetase. Thioguanine, however, is directly converted into TGMP and then into the TGNs by adenosine kinase (Fig. 1; Ref. 2). TIMP and TGMP exhibit similar K_m s for human TPMT (7), but as is true in patients with wild-type TPMT, we found that MeMPN predominated over TGNs after incubation with mercaptopurine, whereas TGN predominated over MeTGN after incubation with thioguanine. As expected, the TPMT+ cells had 9–10-fold higher cytosolic concentrations of methylated nucleotides (MeTGN or MeMPN) compared with the MOCK cells. This was associated with lower concentrations of the TINs (with mercaptopurine treatment) and TGNs (with thioguanine treatment). However, the TGN concentrations were similar between the MOCK and the TPMT+ cells after 24 h of incubation with mercaptopurine. This could be attributable to the high concentrations of the TIMP, which far exceeded the capacity for the TPMT to methylate TIMP and thus left the substrate available for the inosine monophosphate dehydrogenase. Indeed, after 48 h of incubation with mercaptopurine, we observed significantly lower TGN concentrations in TPMT+ cells compared with MOCK cells.

TPMT+ cells produced higher MeMPN levels and were more sensitive to the effects of mercaptopurine (lower IC_{50} and lower viability at 48 h) compared with cells with lower TPMT. This was associated with a faster and more potent inhibition of DNPS and a

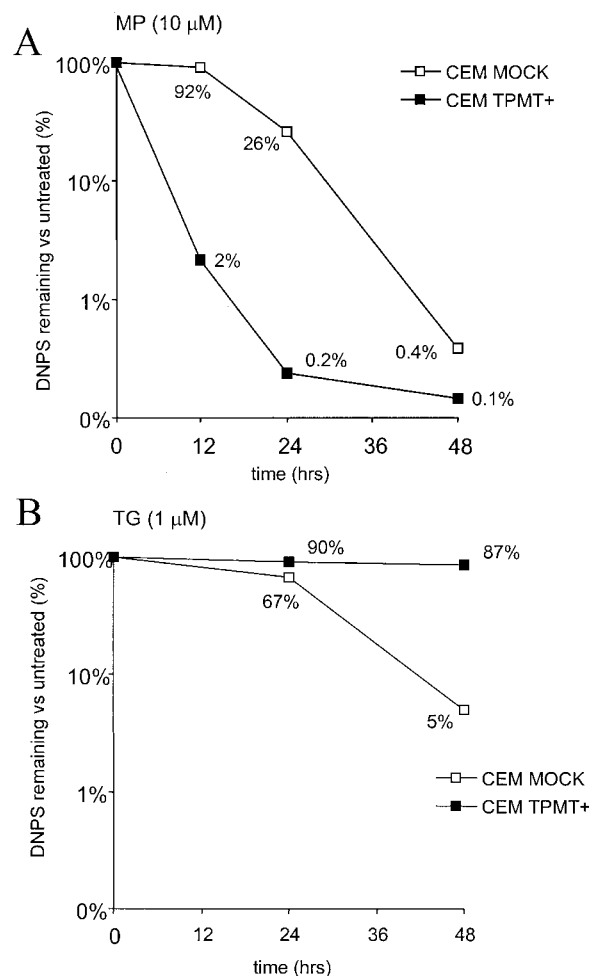


Fig. 6. DNPS inhibition by thioguanine (TG) and mercaptopurine (MP) in CEM cells. A, CEM-MOCK (\square) and CEM-TPMT+ (\blacksquare) cells were treated with $10 \mu\text{M}$ MP, and DNPS inhibition was determined at 12, 24, and 48 h. Results are expressed as the average remaining DNPS compared with untreated controls. Significantly greater inhibition of DNPS was observed in the TPMT+ cells at 12 h ($P = 0.02$) and at 24 h ($P < 0.01$) compared with MOCK cells. No differences were observed at 48 h of treatment ($P = 0.10$). B, CEM-MOCK (\square) and CEM-TPMT+ (\blacksquare) cells were treated with $1 \mu\text{M}$ TG, and DNPS was determined at 24 and 48 h. At each time point, greater inhibition of the DNPS was observed in the MOCK cells compared with TPMT+ cells ($P < 0.05$).

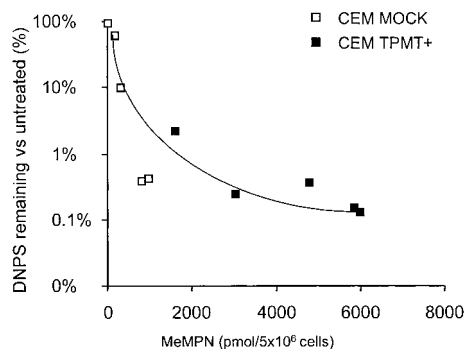


Fig. 7. DNPS inhibition versus MeMPN concentrations in CEM cells. Relationship between DNPS inhibition and concentration of MeMPNs after mercaptopurine treatment. MeMPN concentrations were measured concomitantly with DNPS at 12, 24, and 48 h in the MOCK and the TPMT+ cells. Each point corresponds to the average concentrations for both MeMPN (pmol/5 × 10⁶ cells) and average percentage of DNPS remaining (versus untreated controls). □, experiments with MOCK cells; ■, experiments with TPMT+ cells.

lower percentage of incorporation of deoxythioguanosine into genomic DNA. Thus, our results suggest that MeMPNs contribute to the cytotoxic effects of mercaptopurine.

There are some *in vitro* and *in vivo* data suggesting that methylation of thiopurines may contribute to their cytotoxic or adverse effects. The inhibition of mitogen stimulation in lymphocytes by mercaptopurine was shown to be greater in cells with high TPMT activity (28). Methylmercaptopurine riboside was evaluated as an anticancer drug in its own right (29), has cytotoxic effects *in vitro*, and synergizes with thioguanine (30). MeMPNs are potent inhibitors of phosphoribosylpyrophosphate amidotransferase (the first enzyme in DNPS), with MeTGNs being less potent inhibitors of DNPS (31). The concentration of MeMPNs producing 50% inhibition of DNPS in our *in vitro* model (i.e., 224 pmol/5 × 10⁶ cells) is often achieved *in vivo* in patients having wild-type TPMT activity and receiving oral mercaptopurine (i.e., up to 375 pmol/5 × 10⁶ red blood cells; Ref. 32). Thus, it seems likely that significant DNPS inhibition by MeMPNs can be achieved *in vivo* after oral mercaptopurine and can contribute to the effect of the drug. Methylation of mercaptopurine could also contribute to its effect through the inhibition by methylmercaptopurine riboside of nerve growth factor-activated protein kinase N (33) and angiogenic factors (34). Our results are also consistent with clinical observations. MeMPNs are produced in vast excess compared with TGN in patients with wild-type TPMT activity who received mercaptopurine (32). Although TPMT-deficient patients must be treated with markedly lower doses of mercaptopurine to avoid toxic concentrations of TGN (11), these patients do not form MeMPNs and can tolerate considerably higher TGN concentrations than patients with high TPMT activity (9, 10, 15). Interestingly, patients receiving thioguanine tolerate much higher TGN concentrations than patients receiving mercaptopurine (27); patients treated with thioguanine form no MeMPNs and exhibit a much lower MeTGN/TGN ratio than the MeMPN/TGN ratio achieved with chronic mercaptopurine (9, 27). Together, these data indicate that mercaptopurine could be considered as a “two in one” drug (35), providing a source of TGNs and MeMPNs, and both compounds could contribute to the antiproliferative effects. Two reports (36, 37) have suggested that low TPMT might predispose to the occurrence of secondary acute myeloblastic leukemia after treatment with mercaptopurine, and one of these reports (37) related its risk to either high TGN or high MeMPN concentrations. Thus, there are preclinical and clinical data supporting our finding that MeMPNs are active compounds and should be taken into account during the treatment of acute lymphoblastic leukemia.

Thioguanine treatment in MOCK and TPMT+ cells yielded oppo-

site results compared with mercaptopurine. MOCK cells were more sensitive to thioguanine than TPMT+ cells (lower IC₅₀ and lower viability at 48 h), and this was associated with higher TGN concentrations, higher inhibition of DNPS, and higher incorporation of deoxythioguanosine into DNA. TGNs have been shown to be 4-fold more potent inhibitors of 5-phosphoribosyl amidotransferase than MeTGNs, but 3-fold less potent when compared with MeMPNs (31). Our results are consistent with these *in vitro* findings (31), and we suggest that thioguanine is primarily inactivated by TPMT, with the much higher TGN concentrations in the MOCK cells predominating over any cytotoxic effects of MeTGNs in the TPMT+ cells. It is interesting to note that we did not observe significant cytotoxic effects in the TPMT+ cells despite a 2.0% incorporation of deoxythioguanosine into DNA after 48 h of thioguanine treatment. It is possible that low DNPS inhibition, combined with low deoxythioguanosine incorporation, could lead to low cytotoxic effects. However, other explanations are possible. Thioguanine incorporation into DNA leads to strand breaks (26, 38) and chromatin damage (39), and the mechanism is believed to be related to modification of the conformation and geometry of the DNA. We speculate that the level of deoxythioguanosine incorporation was not high enough to trigger cell death in the TPMT-expressing cells after thioguanine treatment. In contrast, low deoxythioguanosine incorporation after mercaptopurine treatment in the TPMT+ cells may not be as critical, because of the high MeMPN concentrations, which potently inhibited DNPS.

When compared with MOCK cells, the 10-fold higher TPMT activity yielded 9–10-fold higher concentrations of methylated metabolites and 1.6-fold lower deoxythioguanosine incorporation following thioguanine or mercaptopurine treatment. With thioguanine, the lower incorporation of deoxythioguanosine into DNA may explain the ~30% lower viability in the MOCK than in the TPMT+ cells following thioguanine treatment, with MeTGNs putatively contributing little to cytotoxic effects. For mercaptopurine, if only the deoxythioguanosine incorporation into DNA was responsible for its cytotoxic effects, we should have observed lower cytotoxic effects in the TPMT+ cells than in the MOCK cells. However, we observed greater cytotoxic effects in the TPMT+ than in the MOCK cells. We hypothesize that the 10-fold higher concentrations of active MeMPN concentrations formed in the TPMT+ cells must have contributed to the increased sensitivity of the TPMT-expressing cells to mercaptopurine compared with thioguanine, but that the TGN levels also contributed to cytotoxicity.

Because the endogenous substrate of TPMT is unknown, we assessed the possibility that TPMT expression could have modified specific properties of the cells and biased further comparison with thiopurines. However, we found that cells were comparable with regard to parameters such as doubling time, HPRT activity, and DNPS rate. In addition, two drugs that are not methylated by TPMT, methylmercaptopurine riboside and methotrexate, caused equivalent toxicity in the MOCK and the TPMT+ cells. Thus, we made the assumption that the CEM recombinant cells (MOCK and TPMT+) were isogenic and that comparisons of mercaptopurine and thioguanine effects between the cell lines were related primarily to the differing TPMT levels.

Previous *in vitro* studies have demonstrated a higher antiproliferative effect of thioguanine compared with mercaptopurine (40, 41), but clinical studies comparing the effects of these two drugs during the treatment of acute lymphoblastic leukemia found similar antiproliferative effects (27, 42). In our study, cells expressing high TPMT were more sensitive to the effects of mercaptopurine than thioguanine; in contrast, cells expressing low TPMT activity were more sensitive to the effects of thioguanine than mercaptopurine. Thus, the opposite contribution of TPMT to the effects of these drugs could explain why

thioguanine is not superior to mercaptopurine when compared in a population of patients having a polymorphic range of TPMT activities. In addition, 90% of patients are homozygous at the *TPMT* locus and thus have normal or high TPMT activity. Thus, the administration of mercaptopurine in these patients could present an advantage compared with thioguanine because mercaptopurine provides a source of active MeMPNs, which could compensate for the lower TGN levels observed in patients with high TPMT. In contrast, thioguanine administration would be predicted to produce variable responses because of its susceptibility to the highly polymorphic TPMT and the lack of any offsetting activation to MeMPNs.

In conclusion, our results provide new insights into the mechanism of cytotoxicity of thiopurines and suggest a rationale for the choice of mercaptopurine over thioguanine in the treatment of acute lymphoblastic leukemia.

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