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Methylation of Mercaptopurine, Thioguanine, and Their Nucleotide Metabolites by Heterologously Expressed Human Thiopurine *S*-Methyltransferase

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SUMMARY

Thiopurine *S*-methyltransferase (TPMT), a cytosolic enzyme that exhibits genetic polymorphism, catalyzes *S*-methylation of mercaptopurine (MP) and thioguanine (TG), yielding *S*-methylated nucleobases that are inactive, whereas *S*-methylated nucleotides of these thiopurines are cytotoxic. A yeast-based heterologous expression system was therefore used to characterize human TPMT-catalyzed methylation of MP, TG, and their principal nucleotide metabolites [thioinosine monophosphate (TIMP) and thioguanosine monophosphate (TGMP), respectively]. MP, TG, TIMP, and TGMP were all substrates for human TPMT, exhibiting similar Michaelis-Menten kinetic parameters (K_m, 10.6–27.1 μM; V_{max}, 31–59 nmol/min/mg of TPMT). Consistent with these kinetic parameters, human leukemia cells (CEM) incubated for 24 hr with 10 μM MP or TG accumulated significantly higher (2.3-fold, $p = 0.01$) concentrations of methyl-TIMP after MP incubation than methyl-TGMP after TG incubation, due to the 2.7-fold higher concentration of TIMP after MP incubation, compared with TG nucleotides (TGN) after TG incubation. Moreover, intracellular accumulation of TGN was 2.5-fold greater after TG incubation than after MP incubation ($p = 0.01$). These data establish that MP, TG, and their principal nucleotide metabolites are comparable substrates for polymorphic TPMT, and they demonstrate significant differences in the accumulation of active TGN and methylated nucleotides when leukemia cells are treated with MP versus TG.

MP and TG are inactive prodrugs, requiring metabolism to thiopurine nucleotides to exert cytotoxicity. The principal cytotoxic mechanism of MP and TG is generally considered to be via incorporation of TGN into DNA and RNA (1). As depicted in Fig. 1, MP must first be metabolized to TIMP, and then TXMP, before metabolism to TGMP (Fig. 1, *path A*), whereas TG is metabolized directly to TGMP by HPRT (Fig. 1, *path B*). Alternatively, these two drugs can undergo *S*-methylation catalyzed by TPMT or oxidation to thiouric acid by xanthine oxidase (TG must be deaminated to thioxanthine before it is oxidized by xanthine oxidase). However, hematopoietic tissues do not have measurable xanthine oxidase activity (2), leaving TPMT and HPRT as the major enzymes competing for thiopurine metabolism in these cells. TPMT-catalyzed methylation of MP and TG yields the corresponding *S*-methylated thiopurine bases (Fig. 1), both of which are inactive metabolites that are not substrates for HPRT. Alternatively, *S*-methylation of TIMP, the primary metabolite of MP, would give rise to ^{me}TIMP, which is a potent inhibitor of *de novo* purine synthesis (3). Thus, the total intracellular concentration of both methylated and nonmethylated thiopurine nucleotides may be important in determining the ultimate mechanism(s) by which thiopurines are cytotoxic.

TPMT is a cytosolic enzyme that preferentially catalyzes the *S*-methylation of aromatic and heterocyclic thio compounds. This protein has a molecular mass of approximately 28 kDa and is expressed in liver, kidney, erythrocytes, leukocytes, intestine, and a number of other tissues (4–6). TPMT activity in human tissues exhibits genetic polymor-

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ABBREVIATIONS: MP, 6-mercaptopurine; dMP, 6-thiodeoxyinosine; dTG, 6-thiodeoxyguanosine; ^{me}MP, *S*-methylmercaptopurine; ^{me}TG, *S*-methylthioguanine; ^{me}TIMP, *S*-methylthioinosine-5′-monophosphate; r^{me}MP, *S*-methylthioinosine; r^{me}TG, *S*-methylthioguanosine; rMP, 6-thioinosine; rTG, 6-thioguanosine; TG, 6-thioguanine; TGMP, 6-thioguanosine-5′-monophosphate; TGN, total of thioguanine ribo- and deoxyribonucleotides; TIMP, 6-thioinosine-5′-monophosphate; TXMP, 6-thioxanthosine-5′-monophosphate; SAM, *S*-adenosyl-L-methionine; HPRT, hypoxanthine phosphoribosyltransferase; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; BAP, bacterial alkaline phosphatase; TPMT, thiopurine *S*-methyltransferase; ^{me}TGN, methylated thioguanine ribo- and deoxyribonucleotides.

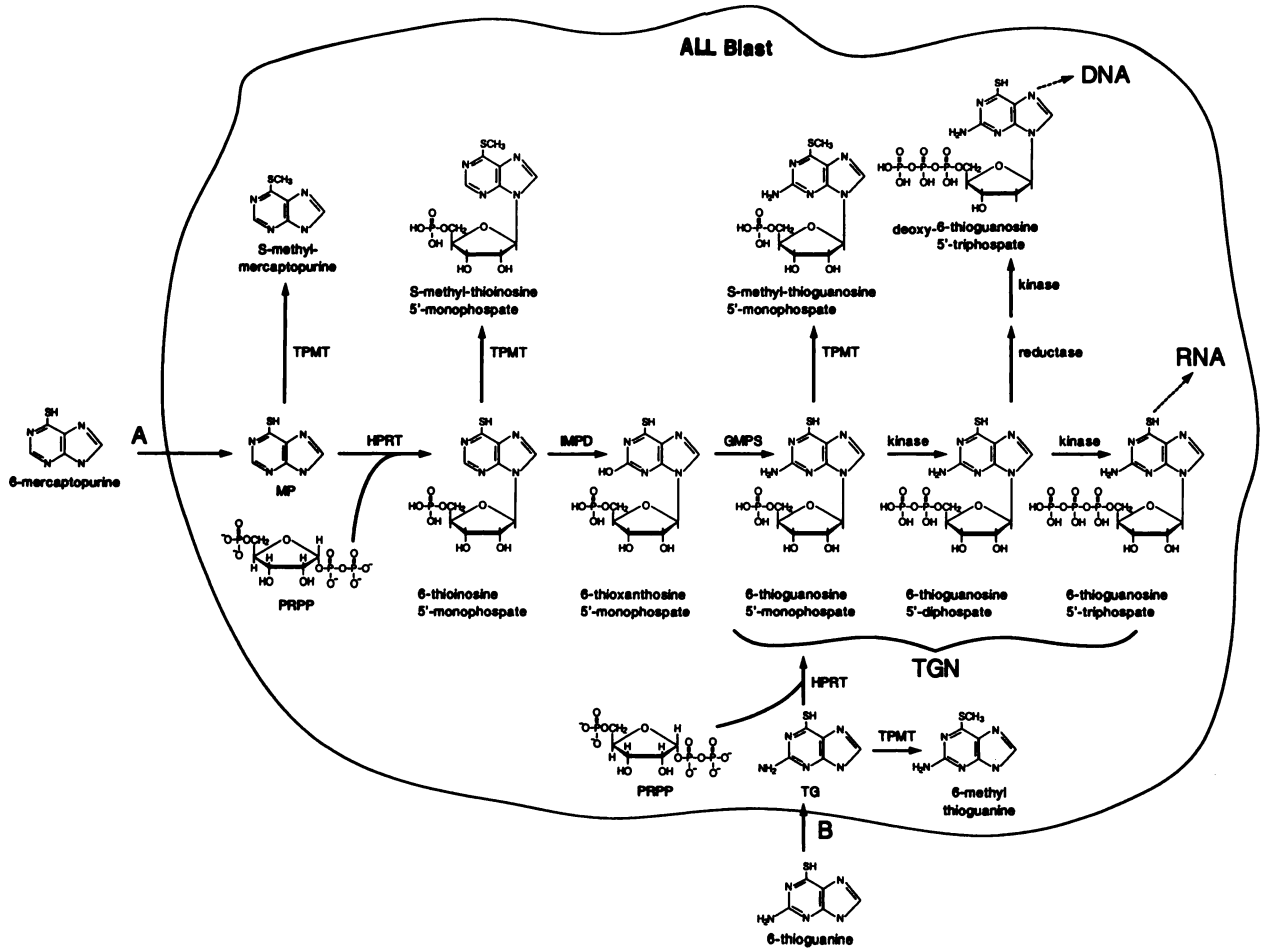

Fig. 1. Metabolism of MP (*path A*) and TG (*path B*) in human acute lymphoblastic leukemia (*ALL*) cells. *IMPD*, inosine monophosphate dehydrogenase; *GMPS*, guanosine monophosphate synthetase; *PRPP*, phosphoribosylpyrophosphate.

phism, with approximately 1 in 300 individuals inheriting TPMT deficiency as an autosomal recessive trait (7, 8). Individual differences in MP metabolism are correlated with the inherited variations in TPMT activity (9), and this can be a significant determinant of the toxicity and antileukemic effects of MP (9–11). A TPMT-encoding cDNA has been cloned and sequenced from a human colon carcinoma cell line (4) and more recently from human liver (5). An inactivating point mutation has been identified at the human TPMT locus and associated with inherited TPMT deficiency, providing initial insights into the molecular basis for this genetic polymorphism (5).

Although MP and TG have been shown to have similar kinetics for TPMT methylation (12, 13), the TPMT-catalyzed methylation of their nucleotide metabolites is not well defined. Given the putative cytotoxic effects of methylated TIMP and/or TGMP, it is important to know the relative affinities of TIMP and TGMP for the polymorphic TPMT enzyme. We used a yeast-based heterologous expression system to characterize human TPMT-catalyzed methylation of MP, TG, and their principal nucleotide metabolites (TIMP and TGMP), as well as other thiopurine derivatives. To estimate the kinetic parameters, we developed a UV-based method for continuous monitoring of the TPMT activity and an HPLC system for analysis of the methylated products of this reaction.

Materials and Methods

Chemicals and reagents. SAM chloride salt, DL-dithiothreitol, Tris·HCl, bovine serum albumin, MP, rMP, dMP, TIMP, ^{me}MP, r^{me}MP, TG, rTG, ^{me}TG, BAP, and proteinase K were obtained from Sigma Chemical Co. (St. Louis, MO). dTG and TGMP were generous gifts of the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (J. Johnson).

HPLC analysis. After TPMT-catalyzed methylation, reaction mixtures containing nucleotides (TIMP and ^{me}TIMP or TGMP and *S*-methylthioguanosine-5′-monophosphate) were incubated with BAP to cleave the 5′-phosphate group; 0.0275 unit of BAP/ml of reaction mixture (for 1 hr at 37°) was added to hydrolyze monophosphate groups, or 0.275 unit of BAP/ml (for 16 hr at 56°) was added to hydrolyze mono-, di-, and triphosphates in the cytosolic fraction of cell lysates. In the absence of BAP incubation, thiopurine nucleosides were consistently undetectable, indicating that all thiopurine nucleosides were formed from nucleotides.

When *S*-methylnucleoside standards were not available (i.e., *S*-methylthiodeoxyinosine, r^{me}TG, and *S*-methylthiodeoxyguanosine), the reaction mixture was treated with perchloric acid and the resulting purine bases were then compared with authentic standards of the corresponding bases; 100 μl of reaction mixture were incubated with 100 μl of 2 M perchloric acid for 1 hr at 100°, the reaction mixture was then neutralized by addition of 2 M Tris·HCl, pH 11, and the content of thiopurine bases was determined by HPLC.

Reaction mixtures were analyzed in duplicate by HPLC, using an

SP8800 pump equipped with an SP8880 autosampler and two SP8773 UV monitors (Spectra-Physics, San Jose, CA). Separation of nucleobases and nucleosides was performed using a Supelco LC-18 column (0.46 × 25 cm), with a linear gradient of methanol (5–40% over 40 min) in 30 mM ammonium phosphate, pH 3.7 (14). Dual-channel monitoring at 290 nm (to detect *S*-methylated derivatives of MP and TG) and 320 nm or 340 nm (to detect thio derivatives of MP and TG, respectively) was carried out using an SP4200 integrator (Spectra-Physics). The identity of *S*-methylated thiopurines was confirmed by comparison with authentic reference compounds (^{me}MP, ^{me}TG, and r^{me}MP) and by characteristic shifts in the UV spectra. In all experiments on TPMT-catalyzed methylation, a control reaction mixture of lysates from yeast without the TPMT cDNA insert was used.

Determination of TIMP, TGN, and their methylated derivatives in cell cytosol. CCRF-CEM cells (human acute lymphoblastic leukemia, T cell lineage, CCL 119; American Type Culture Collection) were used to assess the intracellular metabolism of MP and TG in human leukemia cells. RPMI 1640 medium with 10% fetal bovine serum (BioWhittaker, Walkersville, MD) was used for cell cultures. All experiments were started with an initial concentration of 0.25×10^6 CCRF-CEM cells/ml before addition of the drug. MP or TG dissolved in medium was added as a single dose to achieve a final concentration of 10 μM drug in the culture medium. After 24 hr of incubation with drug, cells were centrifuged (500 × *g* for 5 min at 4°) and washed with Dulbecco's phosphate-buffered saline (2 × 1 ml). Cells (5×10^6) were resuspended in 500 μl of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8), containing 0.5 mg/ml fresh nuclease-free proteinase K, sonicated, and incubated for 30–60 min at 50°. After filtration through a Centricon-3 membrane (1 hr at 6800 × *g* at 21°), the solution was treated with BAP to convert nucleotides to their corresponding nucleosides. The cytosolic TIMP, TGN, ^{me}TIMP, and ^{me}TGN contents (calculated per 1×10^6 cells) were determined from the concentrations of the corresponding nucleosides (i.e., rMP, rTG, r^{me}MP, and r^{me}TG, respectively), quantitated by HPLC analysis of filtrate.

Construction of the yeast expression vector. A cDNA fragment containing the human TPMT coding region was obtained by reverse transcription-PCR, as described earlier (5). The TPMT coding region was cloned into the *Sma*I site of the pGEM7Zf(+) vector and completely sequenced in both directions. Recombinant plasmid containing the TPMT coding region was used as a template for PCR-based site-directed mutagenesis. Primers 5′-CGGATCCAAAATGGATGGTACAGGAACTTCACTTGACATTG-3′ (primer 1) and 5′-CGGAATTCAGGCTTTAGCATAATTTTC-3′ (primer 2) were used to introduce restriction sites as well as to change the ATG context of the initiation codon, to enhance the level of protein synthesis (15). The PCR conditions used were essentially those described previously (16). Authenticity of the PCR product was confirmed by sequencing of the insert from primers complementary to promoter and terminator regions of the pYeDP plasmid and sites within the TPMT cDNA.

Expression in yeast cells. Transformation of the yeast strain 2805 was carried out by lithium acetate/polyethylene glycol treatment (17). Yeast transformed with recombinant expression vectors were grown on galactose-containing medium as described elsewhere (18). Cells were then lysed by sonication, and the homogenate was centrifuged at 34,000 rpm in a 70.1Ti rotor (100,000 × *g*) for 60 min at 4°. The supernatant was diluted 1/1 with glycerol and stored at −70°.

Protein determination and sequencing. Total protein concentrations of yeast lysates were determined by a dye-binding method (19). To determine the concentration of expressed TPMT, yeast lysates were analyzed by electrophoresis and identity was confirmed by sequencing of the amino-terminal peptide of the 31-kDa band corresponding to TPMT (data not shown). To determine the amino-terminal amino acid sequence of the expressed protein, 12–15 μl of lysate (200–250 μg of protein) were applied to a 15% SDS-containing polyacrylamide gel. After the proteins had been transferred to the polyvinylidene difluoride membrane and stained with Coomassie Blue R250, the protein band corresponding to TPMT was excised and sequenced by Edman degradation with the trifluoroacetic acid conversion protocol, using an Applied Biosystems 470A gas-phase protein sequencer equipped with a 120A phenylthiohydantoin analyzer and a 900A control data analysis module, in the Center for Biotechnology at St. Jude Children's Research Hospital. To quantitate the TPMT content in each yeast preparation, the lysate was separated using a 15% SDS-containing gel and was stained with Coomassie Blue R250. The amount of protein in the band corresponding to TPMT was determined by densitometry. Quantitation of the specific protein was carried out using a Bio Image whole-band analyzer, with version 3.1 software (Millipore Corp., Bedford, MA).

TPMT substrate studies by spectrophotometric assay. TPMT-catalyzed methylation was assessed for the following thiopurine compounds: MP, rMP, dMP, TIMP, TG, rTG, dTG, and TGMP. The analysis included continuous spectrophotometric monitoring of methylation and HPLC analysis to confirm the authenticity of product. The reaction mixture, containing 0.1 M Tris·HCl, pH 7.4, 1 mM SAM, and 5–280 μM substrate, was placed in a spectrophotometer cuvette (optical length, 1 or 10 mm), and the reaction was initiated by addition of 3 μl of TPMT preparation to achieve a final TPMT concentration of 3.5 μg/ml (0.1 μM). A DU-40 single-beam spectrophotometer (Beckman, Irvine, CA) equipped with the Soft-Pac module KINETICS was used to determine absorbance at 320 nm for MP, rMP, dMP, and TIMP or at 340 nm for TG, rTG, dTG, and TGMP, in six cuvettes simultaneously, to monitor disappearance of substrate during the incubation at room temperature (21.5°). Readings were taken every 60 sec for 60 min. Apparent K_m and V_{max} values were estimated on the basis of data from experiments performed with different concentrations, as described below. To express the initial rate in micromoles/minute, coefficients of 19.6 absorbance units/μmol for MP and its nucleoside/nucleotide derivatives and 24.8 absorbance units/μmol for TG and its nucleoside/nucleotide derivatives were used.

Estimation of kinetic parameters (V_{max} and K_m). The initial rate of each enzymatic reaction, expressed as decrease in absorbance/minute, was determined from the slope of the linear section of each substrate disappearance versus time curve; the mean of three experiments was used. Nonlinear least-squares regression was used to estimate V_{max} and K_m by fitting a Michaelis-Menten model to the nontransformed substrate disappearance rate (V) versus substrate concentration (S) data for each thiopurine. Equal variances were assumed for the rates of reaction at each substrate concentration. Quasi-Newton and Hooke-Jeeves pattern-moves methods, as implemented in STATISTICA software (StatSoft, Tulsa, OK), were used as nonlinear estimation methods (20). The standard errors of the parameter estimates were computed via finite difference approximation of the second-order partial derivatives (i.e., the Hessian matrix). Lineweaver-Burke plots of reciprocal enzyme velocities ($1/V$) versus reciprocal substrate concentrations ($1/S$) were linear in all cases, consistent with these reactions obeying apparent Michaelis-Menten kinetics under the conditions studied.

Statistical analysis. A two-tailed t test was used to determine whether the model parameter estimates for V_{max} and K_m differed significantly from 0. Differences in the intracellular concentrations of nucleotide metabolites after MP versus TG treatment of leukemia cells were assessed for statistical significance by the Mann-Whitney U test.

Results

The yeast expression system (the episomal expression vector and a peptidase-deficient strain) used to synthesize enzymatically active human TPMT did not possess detectable endogenous TPMT activity, thus exhibiting "zero back-

ground" TPMT activity. Fig. 2 demonstrates the pattern of TPMT protein in lysates from TPMT-expressing yeast, after SDS-polyacrylamide gel electrophoresis analysis. TPMT content in yeast lysates was quantitated using densitometry and was approximately 7% of total protein. Based on the total protein concentration (16.5–17.9 mg/ml in different preparations) and the relative amount of TPMT in lysates (7%), 0.1 μM (3.5 μg/ml) TPMT was used in each reaction mixture to assess kinetic parameters for thiopurine substrates.

The spectrophotometric method for continuous monitoring of the enzymatic reaction is based on the fact that methylation of a thio derivative of a purine base or its nucleoside/nucleotide results in a blue shift in the UV spectrum, thus decreasing the absorbance at λ_{max} for the substrate. The formation of methylated products was confirmed by HPLC analysis of aliquots taken from reaction mixtures throughout the incubation, as exemplified in Fig. 3. Comparison of the HPLC profiles of the methylation products with those of authentic reference standards for ^{me}TG (retention time, 23 min), ^{me}MP (27 min), and r^{me}MP (37 min) demonstrated that recombinant human TPMT expressed in yeast catalyzed methylation of MP and all metabolites evaluated. Because authentic methylated derivatives of dMP, rTG, and dTG were unavailable, these nucleosides were hydrolyzed under acidic conditions to cleave the *N*-glycosidic bond, before HPLC analysis of methylated products. The corresponding methylated thiopurine bases formed were then compared with authentic reference compounds. It was thereby shown that the decrease in absorbance throughout each reaction corresponded to an increase of methylated product, as determined by HPLC.

Using this approach, the rate of enzymatic methylation (V_0) was determined at different substrate concentrations

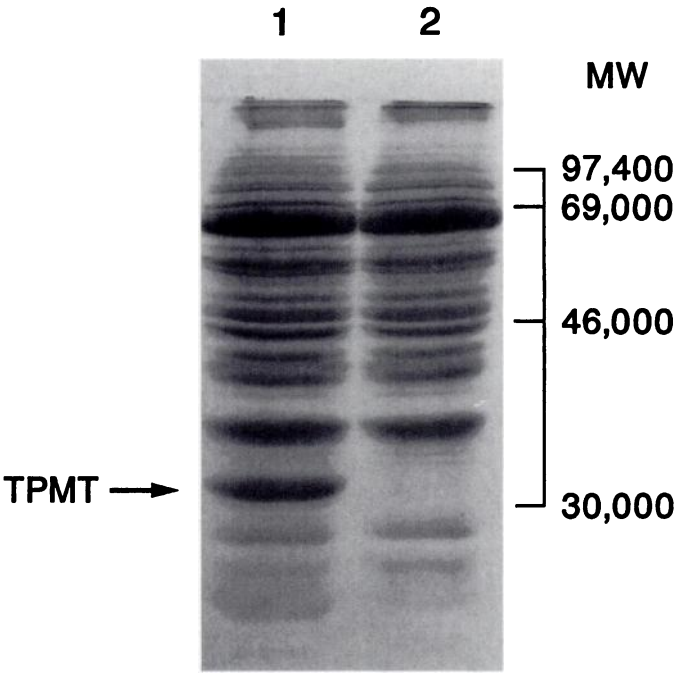

Fig. 2. Analysis of human TPMT-producing yeast in a 15% polyacrylamide gel in the presence of SDS. *Lane 1*, 1 μl of the cytosolic fraction from the yeast producing TPMT; *lane 2*, control (cytosolic fraction from yeast transformed with the expression vector without the TPMT cDNA insert). *Arrow*, position of the band corresponding to TPMT.

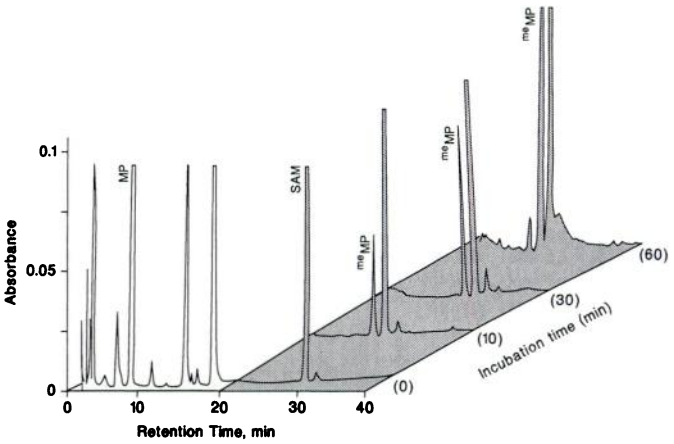

Fig. 3. HPLC analysis of MP methylation products produced by heterologously expressed human TPMT. Each chromatogram represents the analysis of a sample taken at the indicated time during incubation. *Shaded chromatograms*, appearance of ^{me}MP, monitored at 290 nm, after incubation with MP and co-substrate (SAM). *Unshaded chromatogram*, monitored at 320 nm, documenting MP disappearance over time (data not shown).

(5–280 μM) of each thiopurine. Table 1 summarizes V_{max} and K_m values for each substrate, estimated by fitting a Michaelis-Menten model to the untransformed data depicted in Fig. 4. All parameter estimates were significant at $p < 0.05$ (two-tailed t test). All thiopurines evaluated were TPMT substrates, with the two parent drugs and their principal metabolites (TIMP and TGMP) having similar kinetic parameters.

Fig. 5 illustrates the presence of rMP, rTG, r^{me}MP, and r^{me}TG nucleotides in the cytosolic fraction of human leukemia cells (i.e., CEM cells) after *in vitro* incubation for 24 hr with either 10 μM MP or 10 μM TG. These data demonstrate that, with both MP and TG, the major product was the ribonucleotide of the corresponding thiopurine base (i.e., TIMP and TGN), and minor metabolites were methylated products of these ribonucleotides (but not methylated thiopurine nucleobases). The concentration of r^{me}MP nucleotide after MP incubation (280 pmol/5 × 10^6 cells) was >2-fold higher than that of r^{me}TG nucleotide after incubation with TG (120 pmol/5 × 10^6 cells, $p = 0.01$). Likewise, the TIMP concentration after MP incubation (2000 pmol/5 × 10^6 cells) was 2.7 times higher than the TGN concentration after TG incubation (750 pmol/5 × 10^6 cells, $p = 0.01$). Finally, the amount of TG ribonucleotides after incubation with TG (750 pmol/5 × 10^6 cells) was 2.5 times greater than the amount of

TABLE 1

Kinetic parameters of methylation of thiopurines and their derivatives catalyzed by human TPMT expressed in yeast

Parameter estimates are mean ± standard error.

Thiopurine	V_{max}	K_m	V_{max}/K_m
	nmol/min/mg of TPMT	*μM*	*ml/min/mg of TPMT*
MP	48 ± 3	10.6 ± 1.3	4.5
TIMP	31 ± 4	25.7 ± 6.1	1.2
TG	55 ± 5	18.1 ± 3.4	3.0
TGMP	59 ± 2	27.1 ± 1.9	2.2
rMP	89 ± 9	55.1 ± 10.4	1.6
dMP	22 ± 1	12.7 ± 1.0	1.7
rTG	32 ± 3	26.1 ± 4.2	1.2
dTG	120 ± 15	131.4 ± 28.3	0.9

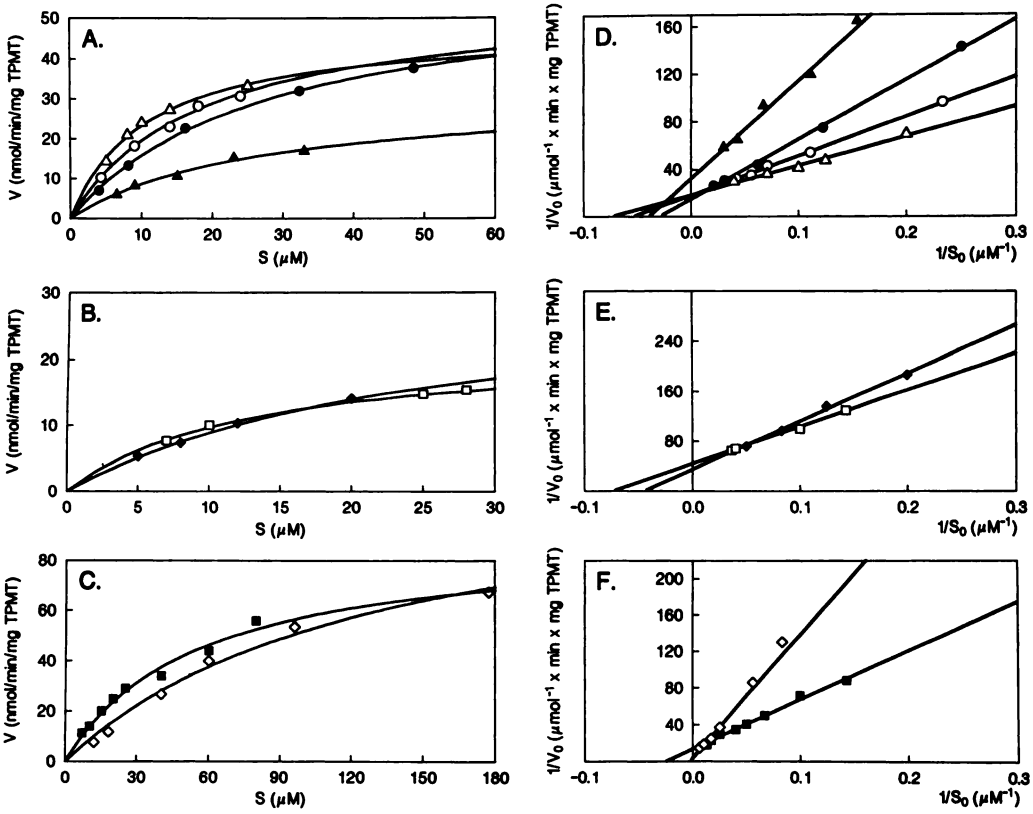

Fig. 4. Kinetics of thiopurine methylation reactions catalyzed by human leukocyte TPMT heterologously expressed in yeast. A-C, Nontransformed substrate disappearance rate (*V*) versus substrate concentration (*S*) plots; *lines*, Michaelis-Menten model simulations. D-F, Lineweaver-Burke plots of the corresponding data. A and D, TIMP (▲), TGMP (●), TG (○), and MP (△); B and E, rTG (◆) and dMP (□); C and F, dTG (◇) and rMP (■).

TG ribonucleotides after incubation with MP (300 pmol/5 × 10^6 cells, $p = 0.01$).

Discussion

TPMT is a cytosolic enzyme that catalyzes *S*-methylation of MP and TG, yielding *S*-methylated nucleobases that are inactive, whereas *S*-methylated nucleotides of these thiopurines are cytotoxic. This study is the first characterization of recombinant human TPMT-catalyzed methylation of MP, TG, and their intracellular metabolites. Using a yeast expression system, the present study establishes that MP, TG, and their principal nucleotide metabolites (i.e., TIMP and TGMP) are all substrates for human TPMT, with similar kinetic parameters (Table 1). This is in contrast to a recent study that reported TGMP to be a poor substrate for purified human kidney TPMT (12). HPLC analysis of the cytosolic fraction of human leukemia cells treated with 10 μM MP revealed the presence of TIMP (the primary product of MP glycosylation), thioxanthine nucleotide, TGN, and ^{me}MP ribonucleotide (i.e., ^{me}TIMP). Treatment with 10 μM TG resulted in accumulation of TGN levels that were 2.5-fold higher than TGN levels after MP treatment ($p = 0.01$) (Fig. 5). After TG exposure, the concentration of ^{me}TGN was 16% of the TGN concentration, which was similar to the ratio of ^{me}MP to TIMP (14%), an intracellular finding consistent with our experiments establishing TIMP and TGMP as comparable substrates for TPMT (Table 1). Therefore, the 2.3-fold higher concentration of ^{me}TIMP after MP incubation (280 pmol/5 × 10^6 cells), compared with ^{me}TGN after TG incubation (120 pmol/5 × 10^6 cells), is apparently due to the 2.7-fold higher concentration of TIMP after MP incubation, compared with TGMP after TG incubation.

It has been shown that MP and TG have different effects on cell cycle progression and GTP pools (21), which could be related to the observed differences in their metabolism and the potency of their methylated nucleotide metabolites. Whereas TPMT-catalyzed methylation of MP or TG bases yields inactive ^{me}MP or ^{me}TG, *S*-methylation of TIMP or TGN yields active nucleotides. ^{me}TIMP is a potent inhibitor of phosphoribosylpyrophosphate-amidotransferase, an enzyme catalyzing the first step in *de novo* purine synthesis (3), whereas ^{me}TGN is about 12-fold less potent as an inhibitor of phosphoribosylpyrophosphate-amidotransferase (22). Moreover, r^{me}MP ribonucleoside was shown to be cytotoxic to human epidermoid cancer cells selected for resistance to MP (23), and r^{me}MP ribonucleoside was shown to be synergistic with either MP (24) or TG (25), consistent with a different mechanism of cytotoxicity for methylated nucleotides of thiopurines.

The principal cytotoxic effect of MP and TG is generally considered to be incorporation of TG nucleotides into DNA and RNA (1), thus necessitating metabolism of both prodrugs to TGN. Therefore, methylation of the parent drugs or their nucleotide intermediates shunts metabolism away from the formation of active TGN. In this regard, Lennard *et al.* (26) have shown that TPMT activity is a significant determinant of TGN accumulation in erythrocytes of patients treated with MP; those with high TPMT activity have lower TGN accumulation. Moreover, patients with inherited TPMT deficiency accumulate very high TGN concentrations (i.e., 10-fold higher) with standard dosages of MP, leading to severe hematopoietic toxicity unless substantial dosage reductions are made (10, 11). Thus, it is apparent that TPMT activity is an important determinant of the metabolism and effects of MP.

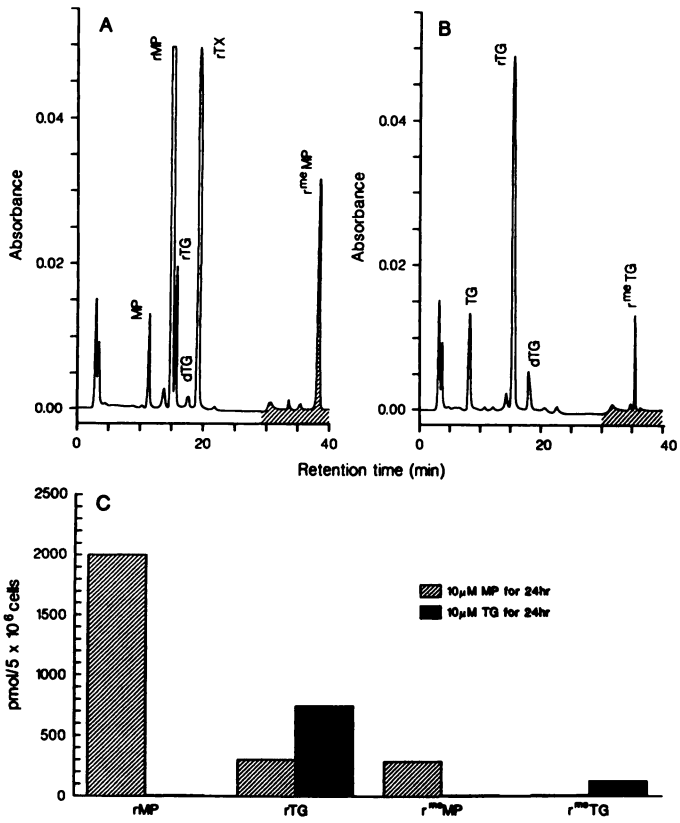

Fig. 5. A and B, Analysis of thiopurine compounds in the cytosolic fraction of human T lineage leukemia cells (CEM) after 24-hr incubation with either MP (A) or TG (B). The nucleosides were liberated from the corresponding nucleotides by incubation with BAP, before HPLC analysis. UV absorbance was monitored at 320 nm and 290 nm (A) or 340 nm and 290 nm (B); *shaded areas*, UV monitoring at 290 nm. C, Relative content of thiopurine nucleotides and their methylated derivatives after incubation with MP versus TG. *rTX*, 6-thioxanthosine.

Among patients who are not TPMT deficient, clinical studies have demonstrated 5-fold higher TGN concentrations in erythrocytes of patients after chronic oral TG treatment, compared with oral MP treatment (27). Although this could be related to MP being more extensively metabolized by xanthine oxidase after oral absorption, in the present study leukemia cells accumulated 2.5-fold higher TGN levels after TG versus MP, in an *in vitro* environment where xanthine oxidase is negligible. MP must be converted to intermediate metabolites (e.g., TIMP and TXMP) before formation of TGN, whereas TG is metabolized directly to TGN, with no intermediate metabolites for TPMT-catalyzed methylation. Because MP, TIMP, TG, and TGMP were comparable substrates for human TPMT, and the intracellular ^{me}TIMP:TIMP and ^{me}TGN:TGN ratios were similar after MP and TG treatment, respectively, it appears likely that mechanisms other than differences in TPMT-catalyzed methylation contribute to lower TGN accumulation after MP. It is possible that less efficient intermediate metabolism of MP (to TIMP to TXMP to TGN) contributes to lower TGN accumulation after MP treatment.

In the present study, all thiopurine derivatives tested were substrates for TPMT-catalyzed methylation of the sulfur atom. Kinetic parameters for these substrates indicate that substitutions in the heterocyclic ring do not significantly change the affinity of these thiopurines for TPMT (Table 1), indicating low specificity of this enzyme toward the purine moiety of these compounds. Although we did not assess TXMP, recent studies indicate that 6-thioxanthine inhibits TPMT activity but is not a substrate for TPMT-catalyzed *S*-methylation (12). In the present study, a decrease in affinity for dTG, compared with nucleobases and rTG, was observed. This may be related to differences in the *syn/anti*-conformation of a ribonucleoside, deoxyribonucleoside, or nucleotide (28).

Thiopurine methylation catalyzed by human TPMT requires the co-substrate SAM as a donor of methyl groups. Concentrations of both substrate and coenzyme were chosen to be 2 (or more) orders of magnitude higher than concentrations of the TPMT protein. However, it is possible that, under certain *in vivo* conditions, intracellular concentrations of SAM could be rate-limiting for methylation of thiopurines and their nucleotide metabolites. It is also important to note that we did not detect any nonenzymatic *S*-methylation, under all conditions tested.

Several previous studies have estimated the kinetic parameters of TPMT-catalyzed methylation of MP, TG, and their derivatives by cell lysates or partially purified TPMT, using radioincorporation or HPLC assays (12, 13, 29). These previous studies have reported discordant results for TPMT methylation of MP (K_m = 83–383 μM), probably due to different methodologies and/or different cell lysate preparations used in these studies. In the present study, we observed a higher affinity of TPMT for both MP (10.6 μM) and TG (18.1 μM), as estimated by continuous monitoring of the reaction catalyzed by human leukocyte TPMT heterologously expressed in yeast. The higher K_m values observed in previous studies, compared with our results, may reflect differences in reaction conditions (e.g., the presence of dimethylsulfoxide in radioassay mixtures and the relatively lower concentration of SAM used in previous assays of TPMT activity) or differences in analytical procedures used to measure product formation (e.g., poor extraction of nucleotide triphosphates with organic solvents). In this regard, it should be noted that the K_m for MP obtained by HPLC analysis of erythrocyte lysates was lower (83 μM) than that obtained by radiochemical assays (320–380 μM) (13, 12, 29). Because the yeast cells do not possess any endogenous TPMT activity and the level of recombinant TPMT expression was relatively high (7% of total protein), we consider our data more comparable to a purified human TPMT preparation. The method we used to continuously monitor methylation and the lower concentrations of all substrates under these conditions obviated the need for dimethylsulfoxide to solubilize substrates and eliminated organic extraction as a variable. Of interest, the V_{max}/K_m ratios for MP, TG, and TIMP (4.5, 3.0, and 1.2 ml/min/mg, respectively) in the present study are comparable to those recently reported (12) using the radiodiochemical assay with partially purified human kidney TPMT (2.3, 1.9, and 1.3 ml/min/mg, respectively), but the V_{max}/K_m ratio we observed for TGMP (2.2 ml/min/mg) is considerably greater than that recently reported with purified human kidney TPMT (0.07 ml/min/mg), the basis of which may be related to methodological differences noted above. Because TGMP is a major active nucleotide metabolite of both MP and TG, its recognition as a comparable substrate for polymorphic TPMT is considered important.

In summary, the two parent drugs and their principal nucleotide products (i.e., TIMP and TGMP) had similar Michaelis-Menten kinetics for TPMT-catalyzed *S*-methylation. Analysis of the cytosolic fraction of human leukemia cells after MP and TG exposure demonstrated intracellular accumulation of methylated metabolites consistent with TIMP and TGMP being comparable substrates for TPMT-catalyzed methylation. Nonetheless, the intracellular concentration of TGN was significantly lower and that of methylated nucleotides was significantly higher after incubation with MP versus TG, due to significantly higher TIMP levels after MP incubation, compared with TGN levels after TG incubation. The principal methylated nucleotide of MP (i.e., ^{me}TIMP) is >10-fold more potent as an inhibitor of *de novo* purine synthesis, compared with ^{me}TGN. It is therefore conceivable that increased inhibition of *de novo* purine synthesis by ^{me}TIMP could "compensate" for the lower accumulation of TGN after MP, compared with TG (30), a possibility that will require further investigation. We have recently established that TPMT activity in leukemia cells of patients with acute lymphoblastic leukemia is highly variable and exhibits genetic polymorphism consistent with that observed in erythrocytes (31). Thus, formation of TGN and *S*-methylated nucleotides in leukemia cells would differ based on the thiopurine administered and the inherited TPMT phenotype, providing additional evidence that the genetic polymorphism of TPMT activity is an important determinant of the metabolism and pharmacological effects of both MP and TG.

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