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Short Communication

PURINE SUBSTRATES FOR HUMAN THIOPURINE METHYLTRANSFERASE

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Abstract—Thiopurine methyltransferase (TPMT) catalyzes the S-methylation of thiopurine drugs such as 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG). A genetic polymorphism regulating TPMT activity in human tissue is an important factor responsible for individual differences in the toxicity and therapeutic efficacy of these drugs. Because of the clinical importance of this polymorphism, we studied 18 purine derivatives, including ribonucleosides and ribonucleotides, as potential substrates for purified human kidney TPMT. Sixteen of the compounds studied were substrates for the enzyme, with K_m values that varied from 29.1 to 1270 μM and with V_{max} values that varied from 75 to 2340 U/mg protein. The thiopurines tested had K_m values that were uniformly lower than were those of the corresponding ribonucleosides or ribonucleotides. 6-Selenopurine derivatives had the lowest K_m values of the compounds studied. Finally, oxidized purines with an OH in the 8-position were methylated by the enzyme, but 2-OH compounds were potent inhibitors of TPMT.

Key words: thiopurine methyltransferase; thiopurines; methylation; pharmacogenetics

6-MP†, 6-TG and azathioprine are used to treat neoplastic and autoimmune diseases, as well as recipients of transplanted organs [1]. The major metabolic pathways for these compounds are S-methylation catalyzed by TPMT (EC 2.1.1.67) [2, 3], oxidation catalyzed by XO (EC 1.2.3.2) [1, 4], and a multi-step metabolic pathway that results in the formation of therapeutically active metabolites, 6-TG nucleotides [5]. TPMT is capable of catalyzing the S-methylation of both heterocyclic and aromatic sulfhydryl compounds [2, 3, 6], and there are large individual variations in TPMT activity in human tissue as a result of a common genetic polymorphism [7–9]. These inherited variations in TPMT activity are a major factor responsible for individual differences in the metabolism, toxicity and therapeutic efficacy of 6-MP and azathioprine [10–12]. Because of the clinical significance of this genetic polymorphism, we set out to compare a series of thio- and selenopurines as substrates for purified human kidney TPMT. The compounds studied included metabolites of 6-MP and 6-TG—either as a result of catabolism to form the corresponding ribonucleotides or oxidation catalyzed by XO. Thiopurines with alkyl groups on the purine structure have been tested as potential antineoplastic agents [13], and selenopurines might be expected chemically to behave in a fashion similar to thiopurines; hence, these compounds were also studied as possible TPMT substrates.

Materials and Methods

TPMT and protein assays. TPMT activity was measured with a modification of the method of Weinshilboum *et al.* [14]. This assay is based on the conversion of substrate to radioactively labeled methylated product, with [^{14}C-methyl]Ado-Met (60 μCi/μmol, NEN-Dupont, Boston, MA) as the methyl donor. Blank samples contained no methyl acceptor substrate. Depending on the pK_a of the substrate studied, the enzyme reaction was terminated either by the addition of 0.5 M borate buffer, pH 10, or 1 N HCl. Reactions performed with hydroxy compounds or ribonucleotides as methyl acceptor substrates were terminated with HCl, while borate buffer was used to stop the reaction for all other substrates. With the exception of experiments performed with ribonucleosides or ribonucleotides, radioactive reaction products were isolated by organic solvent extraction performed with 20% isoamyl alcohol in toluene as described previously [14]. Organic solvent extraction for reactions performed with ribonucleosides was performed with 80% water-saturated isoamyl alcohol in toluene, and 90% water-saturated isoamyl alcohol in toluene was used for reactions performed with ribonucleotide substrates. These solvent compositions had been determined to be optimal by testing solvent mixtures that varied from 100% isoamyl alcohol to 100% toluene. Partition coefficients for each reaction product into the organic solvent phase were determined by back extraction as described previously [14]. One unit of enzyme activity represented the formation of 1 nmol of methylated product/h of incubation at 37°. Protein concentrations were measured by the dye-binding method of Bradford [15] with bovine serum albumin as standard.

TPMT purification. Human kidney TPMT was purified by sequential DEAE Sepharose CL-6B ion exchange and Sephadex G-100 gel filtration chromatography as described elsewhere [16]. Human renal tissue contains two isozymes of TPMT that can be separated by ion exchange chromatography [16]. The "Peak I" isozyme represents over 85% of the total enzymatic activity in human renal preparations, and it was that isozyme, purified approximately 300-fold (sp. act. 1.8 U/μg protein), that was used to perform these experiments.

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† Abbreviations: TPMT, thiopurine methyltransferase; XO, xanthine oxidase; 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; and Ado-Met, *S*-adenosyl-L-methionine.

Table 1. TPMT purine substrate kinetic parameters

Substrates	N	K_m (μM)	V_{max} (U/mg protein)	V_{max}/K_m
1. 6-Selenopurine	6	29.1 ± 0.9	1950 ± 36.8	66.9
2. 6-Selenopurine-riboside	9	51.8 ± 5.9	1990 ± 123.0	38.5
3. 8-Hydroxy-6-MP	6	96.1 ± 2.3	1630 ± 14.7	16.9
4. 7-Methyl-6-MP	6	231.0 ± 15.5	2340 ± 156.0	10.1
5. 6-Selenoguanine-riboside	6	139.0 ± 7.1	1250 ± 23.6	8.96
6. 9-(*n*-Butyl)-6-MP	6	292.0 ± 12.7	1770 ± 79.7	6.07
7. 9-Ethyl-6-MP	6	372.0 ± 12.9	1460 ± 46.2	3.91
8. 6-Hydroxy-8-MP	6	138.0 ± 13.5	324 ± 17.2	2.35
9. 6-MP	6	383.0 ± 7.0	897 ± 8.8	2.34
10. 6-TG	6	557.0 ± 9.9	1080 ± 17.8	1.94
11. 6-MP-riboside-5′-monophosphate	6	1270.0 ± 37.8	1630 ± 24.4	1.28
12. 9-(*n*-Propyl)-6-TG	6	159.0 ± 19.2	169 ± 17.3	1.06
13. 6-MP-riboside	9	1170.0 ± 113.0	641 ± 26.0	0.55
14. 6-TG-riboside	8	761.0 ± 68.0	388 ± 12.3	0.51
15. 6-MP-riboside-5′-triphosphate	6	890.0 ± 56.2	100 ± 3.1	0.11
16. 6-TG-riboside-5′-monophosphate	3	1040.0 ± 70.6	75 ± 0.4	0.07

The nomenclature used in the table emphasizes the relationship of structures of the compounds studied to those of 6-MP and 6-TG. Values for K_m and V_{max} are means ± SEM. N is the number of determinations of each value.

Kinetic analyses. Preliminary experiments performed with each candidate substrate or inhibitor compound involved testing a series of concentrations that differed by several orders of magnitude. Apparent K_m values were then estimated on the basis of data obtained during experiments performed with at least five different substrate concentrations close to the K_m value for that particular compound. K_m values were estimated by the method of Wilkinson [17] with a computer program written by Cleland [18]. Final apparent K_m and V_{max} values reported are averages of all determinations for that compound. Data used to calculate V_{max} values were corrected for the partition coefficients of methylated reaction products into the organic solvent. K_{ii} and K_{is} values for inhibitors were calculated as described by Segel [19].

Materials. 6-MP, 6-MP-riboside, 6-MP-riboside-5′-monophosphate sodium salt, 6-TG, 6-TG-riboside, 6-thioxanthine (2-hydroxy-6-MP), 6-selenopurine, 6-selenoinosine (6-selenopurine-riboside) and 6-selenoguanosine (6-selenoguanine-riboside) were obtained from the Sigma Chemical Co., St. Louis, MO. 6-MP-riboside-5′-triphosphate was purchased from P-L Biochemicals, Inc., Milwaukee, WI. 6-TG-riboside-5′-monophosphate, 8-hydroxy-6-MP and 2,8-dihydroxy-6-MP were gifts from the Burroughs Wellcome Co., Research Triangle Park, NC. 6-Hydroxy-8-MP, 9-(*n*-propyl)-6-TG and 7-methyl-6-MP were purchased from the Chemical Dynamics Corp., South Plainfield, NJ. 9-Ethyl-6-MP and 9-(*n*-butyl)-6-MP were obtained from Flow Laboratories, Inc., McLean, VA. The nomenclature used in parentheses here and that used in Table 1 emphasize the relationship of structures of the compounds studied to those of 6-MP and 6-TG.

Results and Discussion

K_m values, V_{max} values, and ratios of V_{max} to K_m for all purine substrates studied, arranged on the basis of decreasing V_{max}/K_m ratios, are listed in Table 1. The first compounds studied were 6-MP, 6-TG and their respective ribosides and riboside phosphates. The data listed in the table show that 6-MP and 6-TG (compounds 9 and 10, respectively), had very similar K_m, V_{max} and V_{max}/K_m values. K_m values and ratios of V_{max}/K_m for ribonucleosides and ribonucleotides of both of these widely used thiopurine drugs (compounds 11 and 13–16 in Table 1) showed that these derivatives were poorer substrates for the enzyme than were the parent purines. Since oxidation is another metabolic pathway for thiopurines [1, 4], the next set of compounds studied was 2-hydroxy-6-MP, 8-hydroxy-6-MP, and 2,8-dihydroxy-6-MP. 6-Hydroxy-8-MP was also studied. The results showed that 8-hydroxy-6-MP (compound 3 in Table 1) was a good substrate for TPMT. However, neither of the 2-hydroxy derivatives was methylated; therefore, no data for either of those compounds are listed in the table.

The next series of thiopurines studied had alkyl residues at either the 7- or 9-position (compounds 4, 6, 7 and 12 in Table 1). Methylation at the 7-position increased the V_{max}/K_m ratio over that found for 6-MP. Alkylation at the 9-position also increased that ratio slightly for 6-MP derivatives (compounds 6 and 7), while the V_{max}/K_m ratio for the one 6-TG alkyl derivative studied, 9-(*n*-propyl)-6-TG (compound 12), was slightly lower than that of the parent compound. Finally, three selenopurines were studied (compounds 1, 2 and 5 in Table 1). Two of these compounds were the best substrates for TPMT of the purine derivatives studied, with the lowest K_m values and the highest V_{max}/K_m ratios. Once again, the ribonucleoside had a higher K_m value than did its parent purine base.

Since XO can catalyze the 2-hydroxylation of 6-MP after hydroxylation at position 8 [20], we also tested the hypothesis that, although 2-hydroxy-6-MP and 2,8-dihydroxy-6-MP were not substrates for S-methylation, they might be inhibitors of TPMT. Both 2-hydroxy-6-MP and 2,8-dihydroxy-6-MP were found to be inhibitors of the enzyme with 6-MP as the methyl acceptor substrate. Various concentrations of the two co-substrates for the reaction, 6-MP and Ado-Met, were then studied in the presence of a series of concentrations of either 2-hydroxy-6-MP or 2,8-dihydroxy-6-MP. The hydroxylated thiopurines were noncompetitive or "mixed" inhibitors of the enzyme with respect to 6-MP, with K_{is} values of 27 and 95 μM for the 2-hydroxy and 2,8-dihydroxy compounds, respectively. The K_{ii} values were 183 and 340 μM, respectively. Double-reciprocal plots and the "replots" used to calculate K_{is} values for 2-hydroxy- and 2,8-dihydroxy-6-MP are shown in Fig. 1. The 2-hydroxy compound was an uncompetitive inhibitor with respect to Ado-Met, with a K_{ii} value of 99 μM. 2,8-Dihydroxy-6-MP was a noncompetitive inhibitor

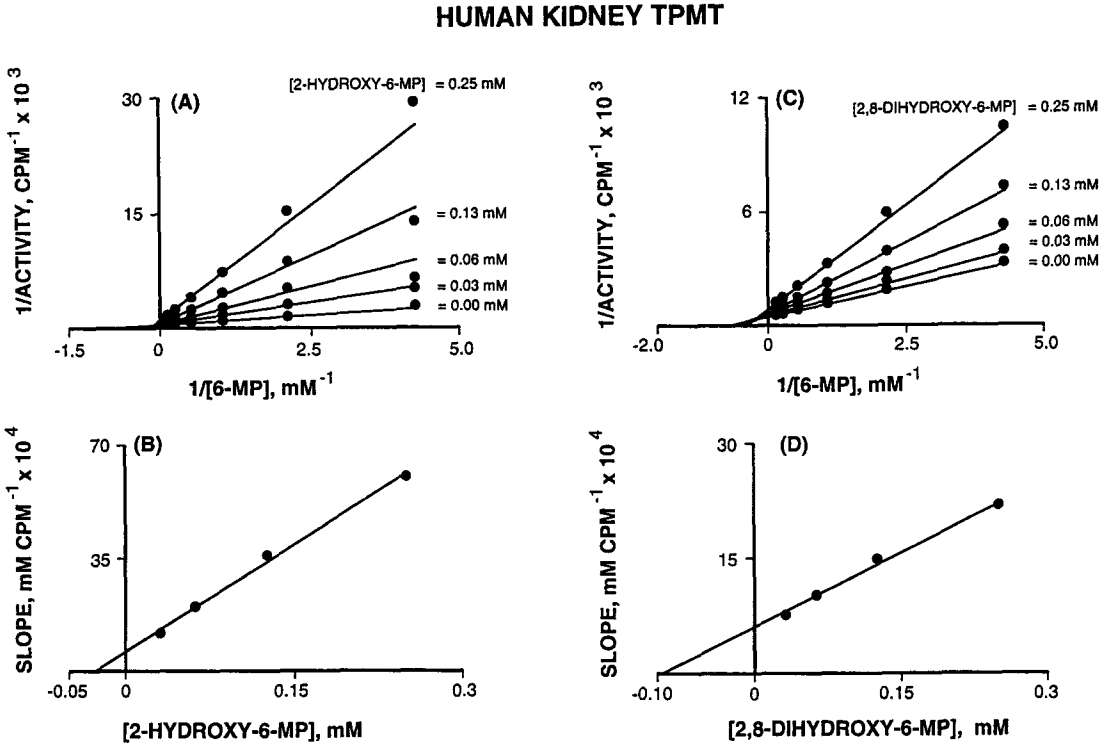

Fig. 1. TPMT inhibition kinetics. (A) Plot of 1/V versus 1/[6-MP] in the presence of various concentrations of 2-hydroxy-6-MP. (B) Plot of slopes calculated from the double-reciprocal plots shown in (A) versus the concentration of 2-hydroxy-6-MP. (C) Plot of 1/V versus 1/[6-MP] in the presence of various concentrations of 2,8-dihydroxy-6-MP. (D) Plot of slopes calculated from the double-reciprocal plots shown in (C) versus the concentration of 2,8-dihydroxy-6-MP.

with respect to Ado-Met, with K_{ii} and K_{is} values of 212 and 402 μM, respectively.

The results of these experiments demonstrated that, *in vitro*, 6-MP and 6-TG are approximately equivalent substrates for TPMT. That observation is of potential clinical importance since it was suggested recently [21] that the TPMT genetic polymorphism might not be so important clinically for patients treated with 6-TG as has been shown to be the case for 6-MP and its "prodrug," azathioprine. The results of our *in vitro* studies would suggest, on the contrary, that the same degree of caution should be exercised in exposing patients with genetically low TPMT activity to 6-TG as is the case with 6-MP. However, the ribonucleosides and ribonucleotides corresponding to 6-MP and 6-TG appear to be worse substrates for TPMT than are the thiopurine bases. We also demonstrated that 2-hydroxy-6-MP and 2,8-dihydroxy-6-MP were potent inhibitors of TPMT. For example, the 27 μM K_{is} value for 2-hydroxy-6-MP is comparable to a K_{is} value of 10 μM for 3,4-dimethoxy-5-hydroxybenzoic acid, one of the most potent TPMT inhibitors described previously [22]. Those observations raise the possibility of an interaction between the two major metabolic pathways for thiopurines, methylation catalyzed by TPMT and oxidation catalyzed by XO—at least for the 2,8-dihydroxy compound, which might be formed *in vivo* [20]. XO activity in human hepatic tissue varies among individuals approximately 4-fold [23]. If oxidized thiopurine metabolites also inhibit TPMT activity *in vivo*, treatment of individuals with relatively higher XO activity with thiopurines might result in inhibition of the methylation pathway, although that hypothesis must be tested experimentally. Finally, the observation that selenopurines are excellent substrates for TPMT raises the issue of the relationship between TPMT and enzymes that catalyze the methylation of selenium compounds [24]. The recent cloning of a cDNA for human TPMT [25] should help make it possible to address those phylogenetic issues in the course of future studies.

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