MOLECULAR PHARMACOLOGY, 24:471–478

Thiopurine Methyltransferase

Aromatic Thiol Substrates and Inhibition by Benzoic Acid Derivatives

LEE C. WOODSON,[1] MATTHEW M. AMES,[2] CYNTHIA DIAS SELASSIE,[3] CORWIN HANSCH,[3] AND RICHARD M. WEINSHILBOUM[1, 4]

Clinical Pharmacology Unit, Departments of Pharmacology and Internal Medicine, and Division of Developmental Oncology Research, Department of Oncology, Mayo Clinic/Mayo Foundation, Rochester, Minnesota 55905, and Department of Chemistry, Pomona College, Claremont, California 91711

Received March 3, 1983; Accepted July 22, 1983

SUMMARY

Thiopurine methyltransferase (TPMT) catalyzes the *S*-methylation of thiopurine and thiopyrimidine drugs. If potent TPMT inhibitors were available, studies of the regulation and properties of this drug-metabolizing enzyme would be facilitated. Each of a series of benzoic acid derivatives tested was found to inhibit purified human kidney TPMT. Concentrations required to inhibit TPMT by 50% ranged from 20 μM for 3,4-dimethoxy-5-hydroxybenzoic acid to 2.1 mM for acetylsalicylic acid. Inhibition was noncompetitive or mixed with respect to both *S*-adenosyl-L-methionine, the methyl donor for the enzyme, and 6-mercaptopurine, the methyl acceptor substrate. Preliminary structure-activity relationship analysis demonstrated that the benzoic acid structure was important for inhibitory activity, and that inhibition was enhanced by the addition of methoxy and/or phenolic hydroxyl groups to the ring. Quantitative structure-activity relationship analysis performed with additional benzoic acid derivatives showed that inhibitory activity could be modeled well by an equation that included the normal Hammett constant and a parameter, π', related to lipophilicity. Several nonheterocyclic aromatic thiol compounds, including thiophenol and thiosalicylic acid, were discovered to be substrates for TPMT. Apparent K_m constants for some of these aromatic thiol compounds were in the nanomolar range, several orders of magnitude lower than those of the thiopurines and thiopyrimidines previously thought to be the only substrates for TPMT. These observations suggested that "aryl thiol methyltransferase" might be a better name than "thiopurine methyltransferase" for this enzyme. Discovery of new classes of inhibitors and substrates for this important drug-metabolizing enzyme has implications for drug metabolism research and for clinical medicine.

INTRODUCTION

TPMT[5] catalyzes the *S*-methylation of thiopurines and thiopyrimidines (1, 2). *S*-Methylation is a major catabolic pathway for these drugs (3). The regulation of TPMT in man and experimental animals has not been studied extensively. However, a common genetic polymorphism has recently been discovered that is responsible for wide variations in human erythrocyte TPMT activity (4). Inherited variations in erythrocyte TPMT activity reflect individual variations of the enzyme activity in other human organs and cells such as the kidney and lymphocyte (5, 6). These observations have raised the possibility that individual differences in TPMT activity might contribute to variations in the therapeutic or toxic effects of thiopurine and thiopyrimidine drugs (4, 7). Interest in TPMT "pharmacogenetics" has also resulted in studies of the regulation of the enzyme in experimental animals (8, 9).

Attempts to study the regulation of TPMT and the properties of the purified enzyme have been hindered by the lack of potent TPMT inhibitors. The observation that tropolone, a COMT inhibitor, was also a noncompetitive inhibitor of purified human kidney TPMT suggested that other COMT inhibitors might affect TPMT

This work was supported in part by National Institutes of Health Grants GM 28157 (R. M. W.), GMC-30362 (C. H.), CA 30054 (M. M. A.), and HL 07269 (L. C. W.).

[1] Clinical Pharamcology Unit, Departments of Pharmacology and Internal Medicine, Mayo Clinic/Mayo Foundation.

[2] Division of Developmental Oncology Research, Department of Oncology, Mayo Clinic/Mayo Foundation. Recipient of National Cancer Institute Research Career Development Award CA 30075.

[3] Department of Chemistry, Pomona College.

[4] Burroughs Wellcome Scholar in Clinical Pharmacology.

[5] The abbreviations used are: TPMT, thiopurine methyltransferase; COMT, catechol-*O*-methyltransferase; 6-MP, 6-mercaptopurine; AdoMet, *S*-adenosyl-L-methionine; Me_2SO, dimethyl sulfoxide; DMHBA, 3,4-dimethoxy-5-hydroxybenzoic acid.

0026-895X/83/060471-08$02.00/0

(2). The present series of experiments was designed to determine whether COMT inhibitors other than tropolone might alter the activity of purified human kidney TPMT. A series of benzoic acid derivatives was found to inhibit TPMT (10). Structure-activity relationship analysis performed with these compounds may make it possible to develop even more potent inhibitors and to characterize the interaction of the inhibitors with the enzyme. It was also discovered that TPMT can catalyze the methylation of nonheterocyclic aromatic sulfhydryl compounds other than the thiopurines and thiopyrimidines previously thought to be the only substrates for the enzyme. Discovery of a new class of TPMT inhibitors and a new class of substrates for the enzyme has potentially important experimental and clinical implications.

MATERIALS AND METHODS

TPMT assay. TPMT activity was measured by the method of Weinshilboum *et al.* (11). The assay is based on the conversion of 6-MP to radioactively labeled 6-methylmercaptopurine with [*methyl*-^{14}C] AdoMet as the methyl donor. Substrate concentrations used in the assay were 3.7 mM for 6-MP and 25 μM for AdoMet. The 6-MP was dissolved in 14 μl of Me_2SO and was added to 86 μl of TPMT in buffer. The pH of the reaction mixture was 6.7. After incubation at 37° for 30 min, the enzyme reaction was terminated by the addition of borate buffer (pH 10). In experiments in which benzoic acid derivatives were used as enzyme inhibitors, the reaction was also terminated by the addition of 1 N HCl to determine whether a methylated product might have been formed. The radioactive reaction product was isolated by solvent extraction performed with 20% isoamyl alcohol in toluene. Radioactivity was measured in a Beckman LS-7500 liquid scintillation counter. Blank samples contained all components of the reaction except the methyl acceptor substrate. The assay procedure has been described in detail previously (2, 11). One unit of TPMT activity represented the formation of 1 nmole of 6-methylmercaptopurine per hour of incubation at 37°.

TPMT inhibitor studies. The ability of a series of compounds to inhibit TPMT was tested. Each compound was dissolved in Me_2SO with or without 6-MP. In all cases, control samples that contained only Me_2SO were also tested. The enzyme assay was then performed exactly as it was for the measurement of TPMT activity. Initially, the effects of at least five different concentrations from 10^{-2} to 10^{-6} M were measured for each compound studied. Once an appropriate concentration range had been determined, a series of concentrations including at least four very near those required to inhibit enzyme activity by 50% (IC_{50} value) were studied. The effect of each concentration was measured in triplicate, and every experiment was repeated at least twice. IC_{50} values were estimated from semilogarithmic plots of the concentration effect curves for inhibition.

TPMT substrate studies. Several aromatic sulfhydryl compounds were tested as possible substrates for TPMT. These assays were identical with the standard enzyme assay performed with 6-MP as substrate except that 6-MP was replaced by the compound to be tested. The purity of each sulfhydryl compound was determined by high-performance liquid chromatography performed with a C18 reverse-phase column with methanol/water (50:50) as the elution solvent and with detection by UV absorption at 254 nm. Under these conditions, all of the compounds studied eluted as a single peak. Initial experiments with each compound were performed with a series of concentrations that differed by several orders of magnitude. Apparent K_m values were then estimated on the basis of data from experiments performed with at least five different concentrations close to the K_m value for the compound. The effect of each concentration was measured in triplicate, and each experiment was performed a minimum of two times.

Protein assay. Protein concentrations were measured by the dye-binding method of Bradford (12) with bovine serum albumin as a standard.

Purification of TPMT. Human kidney TPMT was purified as described in detail elsewhere (2). Briefly stated, human renal tissue was obtained from patients undergoing clinically indicated nephrectomies. The kidneys were obtained under guidelines established by the Mayo Clinic Human Studies Committee. The renal tissue was homogenized, and supernatant fluid was obtained after centrifugation at 100,000 × *g* for 1 hr. The supernatant was subjected to ammonium sulfate precipitation, ion exchange chromatography with DEAE-A25, and gel filtration chromatography through Sephadex G-100. These procedures resulted in a greater than 300-fold purification of the enzyme as compared with the activity present in the supernatant after centrifugation at 100,000 × *g*. The purified TPMT contained no COMT activity. Other characteristics of the purified enzyme have been described elsewhere (2).

Kinetic analysis. Michaelis-Menten constants were estimated by the method of Wilkinson (13) with a computer program written by Cleland (14). K_{ii} and K_{is} values were calculated as described by Segel (15). A Hewlett-Packard 9845B computer was used for these calculations.

Quantitative structure-activity relationship analysis. Quantitative structure-activity relationship analysis was performed by the method of Hansch (16, 17).

Materials. [^{14}C-*methyl*]AdoMet (specific activity 58 mCi/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). AdoMet-HCl, allopurinol, crystalline bovine serum albumin, dithiothreitol, benzoic acid, gallic acid, vanillic acid, thiophenol, DEAE-A25 ion exchange resin, and Sephadex G-100 were purchased from Sigma Chemical Company (St. Louis, Mo.). Acetylsalicylic acid; *m*-anisaldehyde; *o*-, *m*-, and *p*-anisic acid; isophthalic acid; phthalic acid; salicylic acid; thiosalicylic acid; 4-acetamidothiophenol; vanillin; and veratric acid were purchased from Aldrich Chemical Company (Milwaukee, Wisc.). α-Mercaptoacetanilide was obtained from Pfaltz and Bauer Inc. (Stanford, Conn.). 3,4-Dimethoxy-5-hydroxybenzoic acid was purchased from ICN Pharmaceuticals (Plainview, N. Y.). Pyrogallol was obtained from Fisher Scientific (Pittsburgh, Pa.). Dye reagent for the protein assay was purchased from Bio-Rad Laboratories (Richmond, Calif.). 5-Hydroxy-3-mercapto-4-methoxybenzoic acid and a series of other benzoic acid derivatives were kindly donated by Dr. R. T. Borchardt, The University of Kansas (Lawrence, Kan.).

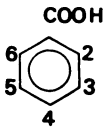
RESULTS

Inhibition of TPMT by benzoic acid derivatives. Tropolone, a COMT inhibitor, has been reported to inhibit TPMT (2). Therefore, two other COMT inhibitors, pyrogallol and DMHBA, were tested to determine whether they could also inhibit the enzyme. Pyrogallol at concentrations as high as 20 mM was not an inhibitor, but DMHBA at a concentration of 20 μM caused a 50% reduction in TPMT activity. Since DMHBA is a derivative of benzoic acid, a series of benzoic acid compounds was screened for ability to inhibit purified human kidney TPMT. Each of the eight compounds tested inhibited the enzyme (Table 1). However, there was great variation in the potency of the compounds. Concentrations required to inhibit TPMT activity by 50% (IC_{50} values) ranged from 20 μM for DMHBA to 2.1 mM for acetylsalicylic acid (Table 1).

An experiment was performed to determine whether the apparent inhibition of TPMT by these compounds might be related to interference with the organic solvent extraction procedure used in the enzyme assay. Each inhibitory compound was added to the TPMT reaction mixture after termination of the enzyme assay but prior to the organic solvent extraction step. The concentration of each compound used was approximately that of its IC_{50} value. Under these conditions, TPMT activity varied

TABLE 1

Structures and IC_{50} values for benzoic acid derivatives that were initially evaluated for their ability to inhibit TPMT



Compound	IC_{50}	Substituents other than hydrogen			
		2	3	4	5
	mM				
DMHBA	0.02	—	OCH_3	OCH_3	OH
Veratric acid	0.10	—	OCH_3	OCH_3	—
m-Anisic acid	0.20	—	OCH_3	—	—
Salicylic acid	0.28	OH	—	—	—
Vanillic acid	0.48	—	OCH_3	OH	—
Benzoic acid	1.10	—	—	—	—
Gallic acid	1.70	—	OH	OH	OH
Acetylsalicylic acid	2.10	$OCOCH_3$	—	—	—

from 98% to 108% of control values. These results demonstrated that the inhibitors had to be present during the enzyme reaction itself to alter the enzyme activity.

Preliminary structure-activity relationship analysis. All of the compounds tested initially as TPMT inhibitors were benzoic acid derivatives, but the most potent compounds, DMHBA and veratric acid, had methoxy substituents. The structure of DMHBA also included a phenolic hydroxyl group. Therefore, a series of compounds related structurally to DMHBA and veratric acid were studied in a preliminary attempt to evaluate structure-activity relationships. Inhibitory activities for these compounds were expressed as IC_{50} values estimated from concentration-effect curves. Of the three types of ring substituents present on DMHBA, the carboxyl group seemed most important for inhibition of TPMT. The IC_{50} value for benzoic acid was 1.1 mM (Table 1). In the absence of a carboxyl group, neither phenolic hydroxyl groups in compounds such as phenol, catechol, or pyrogallol nor methoxy groups in compounds such as anisole conferred significant TPMT inhibitory activity. Of the non-benzoic acid phenol and methoxy compounds tested initially, only veratrole, a dimethoxy derivative, showed detectable inhibition. However, veratrole's IC_{50} value of 10 mM was an order of magnitude greater than that of benzoic acid.

A series of preliminary experiments was then conducted to study structure-activity relationships for benzoic acid inhibitors of TPMT. The first set of experiments (Table 2, Group A) demonstrated that compounds with more than one carboxyl group were less active than benzoic acid itself. The IC_{50} values for phthalic acid and isophthalic acid were twice that of benzoic acid (Table 2, Group A). The importance of the carboxyl group for TPMT inhibition was confirmed by comparison of the activities of benzoic acid derivatives with those of an analogous series of benzaldehyde compounds. Although all of the benzaldehydes studied were TPMT inhibitors, in each case the IC_{50} value of the corresponding benzoic acid derivative was considerably lower than that of the

TABLE 2

Preliminary structure-activity relationship analysis for TPMT inhibitors

See text for a detailed description of experimental Groups A–D.

Group	Compound	IC_{50}	Substituents other than hydrogen				
			1	2	3	4	5
		mM					
A	Benzoic acid	1.1	COOH	—	—	—	—
	Phthalic acid	2.3	COOH	COOH	—	—	—
	Isophthalic acid	2.3	COOH	—	COOH	—	—
B	*p*-Anisic acid	0.3	COOH	—	—	OCH_3	—
	p-Anisaldehyde	7.9	CHO	—	—	OCH_3	—
	Vanillic acid	0.5	COOH	—	OCH_3	OH	—
	Vanillin	1.9	CHO	—	OCH_3	OH	—
	Veratric acid	0.1	COOH	—	OCH_3	OCH_3	—
	Dimethoxybenzaldehyde	8.8	CHO	—	OCH_3	OCH_3	—
C	Vanillic acid	0.5	COOH	—	OCH_3	OH	—
	Homovanillic acid	5.5	CH_2COOH	—	OCH_3	OH	—
	Gentisic acid	0.5	COOH	OH	—	—	OH
	Homogentisic acid	5.0	CH_2COOH	OH	—	—	OH
D	Anisic acid						
	o-	>10	COOH	OCH_3	—	—	—
	m-	0.2	COOH	—	OCH_3	—	—
	p-	0.3	COOH	—	—	OCH_3	—

comparable benzaldehyde (Table 2, Group B). Exactly the opposite relationship between benzoic acid derivatives and benzaldehydes was found when similar compounds were tested as COMT inhibitors (18). Separation of the carboxyl group from the benzene ring by even a single carbon atom greatly reduced the inhibition of TPMT. The IC_{50} values of vanillic acid and gentisic acid were approximately 1 order of magnitude lower than those of homovanillic acid and homogentisic acid (Table 2, Group C). Finally, as expected, the relative positions of substituents on the ring played an important role in their effects on TPMT inhibition. When three different isomers of anisic acid were studied, the rank order of their potencies related to the position of the methoxy group was $m \geq p \gg o$ (Table 2, Group D). Although this series of experiments was only preliminary, it did serve as the basis for a more quantitative structure-activity relationship study. The results of the quantitative study will be described subsequently.

Reversibility of TPMT inhibition. Experiments were performed with two TPMT inhibitors, DMHBA and salicylic acid, to determine whether inhibition of the enzyme was reversible. Salicylic acid was chosen for study because of the frequent clinical use of this compound, both alone and as its acetyl ester. DMHBA was chosen because it was the most potent of the benzoic acid inhibitors initially studied. Purified TPMT was incubated for 15 min at 37° with both of these compounds, and the mixtures were then rapidly dialyzed by three cycles of dilution and concentration in an Amicon pressure concentrator with a PM 10 membrane. This method of dialysis was chosen because the purified enzyme was unstable during overnight dialysis against a large volume of dialysate. Incubation with salicylic acid inhibited TPMT activity approximately 50% before dialysis, but 99% of the activity was recovered after dialysis as compared with a control sample that was subjected to incubation and pressure dialysis without the addition of salicylic acid (Table 3). When a similar experiment was performed with DMHBA, inhibition was not reversed by dialysis (Table 3). The experiment was repeated several times with similar results. There was no obvious explanation for the difference in behavior between salicylic acid and DMHBA.

Kinetics of TPMT inhibition. Kinetic analysis of the inhibition of TPMT by benzoic acid derivatives was performed with respect to the methyl donor, AdoMet, and the methyl acceptor substrate, 6-MP. These experiments were performed by measurement of enzyme activity in the presence of varying concentrations of inhibitors and cosubstrates. K_{ii} and K_{is} values calculated from these data are shown in Table 4. Figure 1 shows double-inverse plots of the effects of the most potent inhibitor, DMHBA, on TPMT activity measured in the presence of varying concentrations of 6-MP and AdoMet. The kinetic characteristics of inhibition by DMHBA were typical of those of the other inhibitors studied. Noncompetitive or mixed inhibition was observed with respect to both the methyl donor and the methyl acceptor substrate for each of the benzoic acid inhibitors. In each case, increasing concentrations of inhibitor resulted in increased apparent K_m values and decreased V_{max} values.

Quantitative structure-activity relationship analysis. Results of the preceding experiments served as the basis for quantitative structure-activity relationship studies. These studies were made possible by the availability of a series of benzoic acid derivatives synthesized as COMT inhibitors by Dr. R. T. Borchardt, of the University of Kansas (18). The structures of these compounds and their IC_{50} values for TPMT inhibition are shown in Table 5 with several parameters that were used in the quantitative structure-activity relationship analysis. Since only very small quantities of these compounds were available, full kinetic experiments were not possible, and the quantitative structure-activity relationship evaluation was based on IC_{50} values. These studies have utilized the approach developed by Hansch (16, 17) in which an attempt is made to model mathematically the results of experiments with inhibitors by taking into account π, a factor related to the hydrophobic or hydrophilic characteristics of substituents added to a given structure, as well as the Hammett constants of the substituents (19). The use of the Hammett constants takes into account the electron withdrawing or electron donating characteristics of the substituents. The ultimate goal of the experiments was to determine both whether it might be possible to predict the behavior of untested compounds as potential inhibitors of TPMT and to attempt to study the nature of the interaction between enzyme and inhibitor.

When this approach was used for the substituted ben-

TABLE 3

Reversibility of inhibition of TPMT by salicylic acid and DMHBA

TPMT was incubated with each compound for 15 min at 37°. An aliquot was then subjected to three cycles of pressure dialysis followed by dilution with buffer. "Recoveries" of enzyme activities are expressed for each column separately as a percentage of control enzyme activity incubated without inhibitor; e.g., the 99% figure for salicylic acid in the right-hand column represents 99% of the 85% of activity that remained after dialysis alone.

	No dialysis	Dialysis
Control	100%	85%
Salicylic acid, 1 mM	54%	99%
DMHBA, 20 μM	50%	55%

TABLE 4

Kinetic constants for inhibition of TPMT by benzoic acid derivatives

K_{ii} and K_{is} values represent the calculated concentrations of inhibitor required to double the intercept or the slope, respectively, of double-reciprocal plots.

Compound	6-MP as variable substrate		AdoMet as variable substrate	
	K_{ii}	K_{is}	K_{ii}	K_{is}
	mM	mM	mM	mM
DMHBA	0.019	0.010	0.014	0.066
Veratric acid	0.110	0.120	0.110	0.120
m-Anisic acid	0.17	0.20	NT[a]	NT[a]
Salicylic acid	0.53	0.22	0.13	NL[b]
Vanillic acid	0.56	0.55	NT[a]	NT[a]
Benzoic acid	0.87	NL[b]	NL[b]	4.4
Acetylsalicylic acid	5.40	1.40	1.90	2.5

[a] Not tested with respect to that substrate.

[b] Not linear with regard to the indicated parameter.

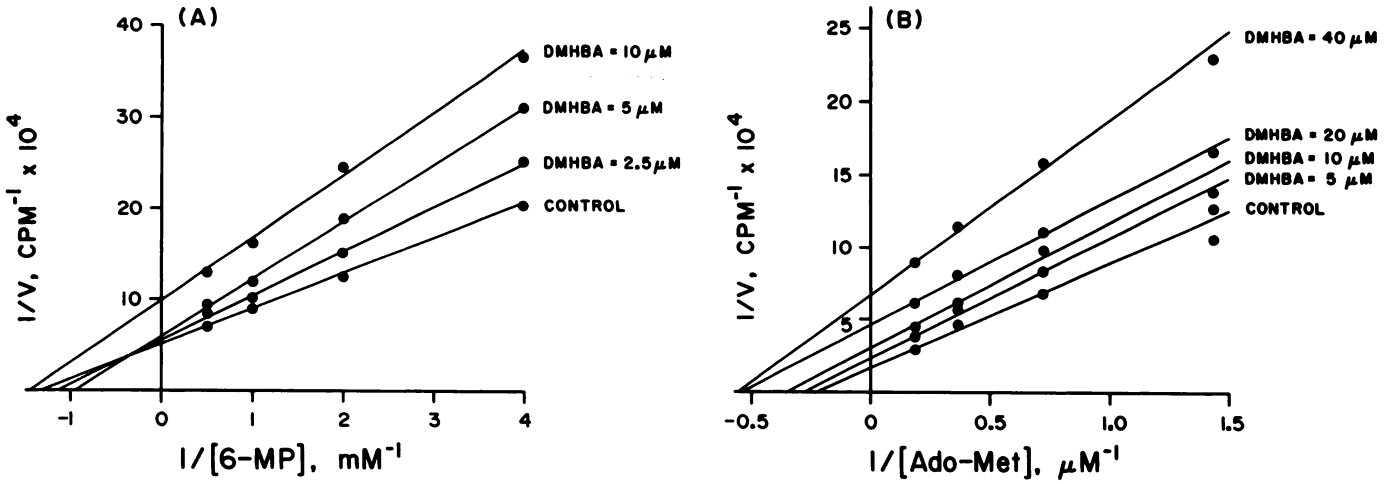


FIG. 1. *Kinetics of TPMT inhibition by DMHBA*

The double-inverse plots show the effects of different concentrations of DMHBA on TPMT activity when 6-MP and AdoMet concentrations were varied. Each point represents the average of three determinations.

zoic acid compounds listed in Table 5, it was apparent from preliminary calculations that the hydrophobic effect modeled by the parameter π' played an important role for only one of the two possible *m*-substituents, that which was assigned to position 3. The more hydrophilic of the two *m*-substituents was assigned to position 5 in Table 5. The π factor for these substituents was ignored in the derivation of the equations described subsequently. It was evident that $\sum \sigma$, in which σ was the normal Hammett constant, was also of importance for inhibition of TPMT. The variation of 4-substituents for the series of compounds studied was limited, since 10 of the 12 congeners had a 4-OCH_3 moiety. For that reason, it could not be determined whether the 4-substituents had a hydrophobic or a steric role in the interaction of the inhibitor with the enzyme. Therefore, parameterization of 4-substituents and the more hydrophilic of the two *m*-substituents was neglected except for σ. It was then possible to derive the following equations utilizing π' and $\sum \sigma$:

$$pI_{50} = 1.54(\pm 0.97)\pi_3' + 4.04(\pm 0.49) \quad (1)$$
$$n = 12, \quad r = 0.746, \quad s = 0.724, \quad F_{1,10} = 12.5$$

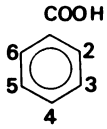
$$pI_{50} = 1.25(\pm 0.64)\pi_3' + 2.20(\pm 1.24)\sum \sigma + 4.05(\pm 0.32) \quad (2)$$
$$n = 12, \quad r = 0.917, \quad s = 0.457, \quad F_{1,9} = 16.1$$

In these equations, n represents the number of data points used to derive the equations, r is the correlation coefficient, s is the standard deviation, and F is the F

TABLE 5

Quantitative structure-activity relationship analysis for inhibition of TPMT by benzoic acid derivatives

pI_{50} values are the $-\log_{10}$ of IC_{50} values. Calculated pI_{50} values were computed by the use of Eq. 2 (see text for details). π'_3 represents the π constant for *m*-substituents. When there were two *m*-substituents, only the more hydrophobic one was assigned a π value. $\sum\sigma$ refers to the sum of the constants for all substituents. π'_3 and $\sum\sigma$ values were used in Eq. 2 to calculate pI_{50} values.



Compound no.	Substituents other than hydrogen			IC_{50}	pI_{50} observed	pI_{50} calculated	ΔpI_{50}	π'_3	$\sum\sigma$
	3	4	5						
				μM					
1	I	OCH_3	OH	1.2	5.92	5.89	0.03	1.12	0.20
2	Cl	OCH_3	OH	2.6	5.59	5.42	0.17	0.71	0.22
3	Br	OCH_3	OH	3.0	5.52	5.65	−0.13	0.86	0.24
4	NO_2	OCH_3	OH	9.0	5.05	4.94	0.11	−0.28	0.56
5	OCH_3	OCH_3	OH	20	4.70	3.96	0.74	−0.02	−0.03
6	—	OCH_3	OCH_3	100	4.00	3.72	0.28	0.00	−0.15
7	$N(CH_3)_2$	OCH_3	OH	125	3.90	3.62	0.28	0.18	−0.30
8	—	—	OCH_3	200	3.70	4.32	−0.62	0.00	0.12
9	—	OCH_3	—	300	3.54	3.46	0.08	0.00	−0.27
10	—	OH	OCH_3	500	3.32	3.50	−0.18	0.00	−0.25
11	OH	OCH_3	OH	600	3.22	3.15	0.07	−0.67	−0.03
12	—	OCH_3	OH	1300	2.89	3.72	−0.83	0.00	−0.15

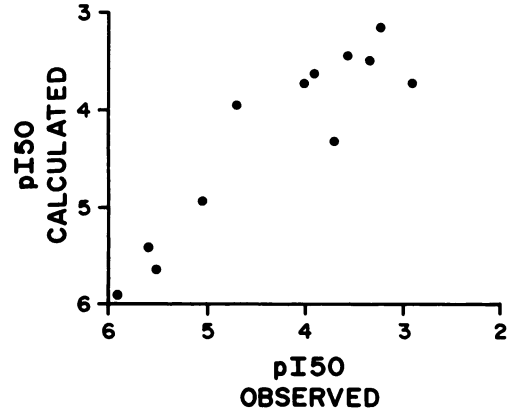


FIG. 2. *Observed and calculated pI_{50} values for inhibition of TPMT by a series of benzoic acid derivatives*

pI_{50} values are $-\log_{10}$ of IC_{50} values. Calculated values were computed with an equation derived by quantitative structure-activity relationship analysis. See text for details.

statistic. Each of the equations was highly significant ($F_{1,9}\alpha 0.01 = 10.6$; $F_{1,8}\alpha 0.001 = 15.4$). The figures in parentheses represent the 95% confidence intervals. Equation 1 was the best single-variable equation. There was a range of 1000 for the IC_{50} values shown in Table 5, and Eq. 2 predicted the TPMT inhibitory activity of the compounds within a factor of approximately ±2.5. The behavior of the two examples of monosubstituted congeners was predicted by Eq. 2 as well as that of the di- or tri-substituted benzoic acid derivatives. A plot of the experimentally observed pI_{50} values versus those calculated by the application of Eq. 2 is shown in Fig. 2.

Although there were only three types of substituents in position 5, the large difference among π for OH (−0.67), for H (0.0), and for OCH_3 (−0.02) meant that, if both 3- and 5-substituents could contact the enzyme simultaneously, a satisfactory correlation would not be obtained by omitting π for one substituent. The positive coefficient with $\sum \sigma$ shown in Eq. 2 demonstrated that electron-attracting substituents favored binding, and thus inhibition. The π values shown in Table 5 are those of the benzene system. That is, $\pi = \log P_{C_6H_5-X} - \log P_{C_6H_6}$, where P is the octanol/water partition coefficient. The fact that only small quantities of these compounds were available made it impossible to determine π' values experimentally. Although experimentally determined π values might have improved the calculated correlation slightly, the effect would probably have been negligible and would certainly not have altered the over-all conclusions of the studies.

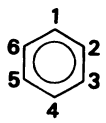
Aromatic sulfhydryl compounds as TPMT substrates. TPMT has previously been reported to catalyze the *S*-methylation of only thiopurines, thiopyrimidines, and extremely high concentrations of 2-mercaptoethanol (apparent K_m of 168 mM) (1, 2). In the course of these studies of TPMT inhibitors, five additional aromatic thiol compounds were found to be substrates for the purified human kidney enzyme (Table 6). Although each of these compounds was aromatic, none was heterocyclic. Of perhaps more importance, all but one of them had a significantly lower apparent K_m constant than has been reported for more "traditional" TPMT substrates such as 6-MP, 6-thioguanine, and 2-thiouracil. The apparent K_m constants shown in Table 6 may be compared with values of 300 μM, 550 μM, and 2000 μM for 6-MP, 6-thioguanine and 2-thiouracil, respectively (2), values calculated from the results of experiments performed with the same purified human kidney TPMT preparation. One additional cyclic thiol, the deacetylated sulfhydryl metabolite of spironolactone, 7-α-thiospirolactone, was tested as a potential substrate for TPMT. In man, this metabolite is probably converted to an *S*-methyl derivative (20). There was no evidence that 7-α-thiospirolactone was able to serve as a substrate for purified human kidney TPMT even at concentrations as high as 10 mM. That observation is not surprising in light of a recent report (21) that the sulfhydryl metabolite of spironolactone is a substrate for thiol methyltransferase (EC 2.1.1.9), a membrane-associated activity that differs from TPMT in subcellular location, in substrate specificity, in sensitivity to inhibitors, and in regulation (1, 2, 4, 21).

DISCUSSION

The experiments described here have demonstrated that a variety of benzoic acid derivatives are inhibitors of TPMT. Preliminary structure-activity analysis indicated that the carboxyl group was important for TPMT inhibition and that displacement of that group from the

TABLE 6

Structures and apparent K_m constants of aromatic nonheterocyclic substrates for TPMT



Compound	Substituents other than hydrogen					Apparent K_m
	1	2	3	4	5	
						μM
α-Mercaptoacetanilide	$NHCOCH_2SH$	—	—	—	—	280
Thiosalicylic acid	COOH	SH	—	—	—	7.3
4-Acetamidothiophenol	SH	—	—	$NHCOCH_3$	—	5.5
Thiophenol	SH	—	—	—	—	0.36
5-Hydroxy-3-mercapto-4-methoxybenzoic acid	COOH	—	SH	OCH_3	OH	0.29

aromatic ring on a carbon side-chain decreased inhibition. Inhibition was enhanced by the addition to the aromatic ring of methoxy and/or phenolic hydroxyl substituents. Quantitative structure-activity relationship studies demonstrated that it was possible to model mathematically the results of experiments performed with a series of inhibitors, and, thus, to predict the behavior of benzoic acid derivatives as TPMT inhibitors. Finally, the unexpected observation was made that nonheterocyclic aromatic thiols were substrates for TPMT. Apparent K_m constants for some of these compounds were several orders of magnitude lower than those of any thiopurine or thiopyrimidine substrate previously studied.

The discovery of potent new TPMT inhibitors has both experimental and clinical implications. The availability of potent enzyme inhibitors may make it possible to study the effects of TPMT inhibition on the response to and toxicity of thiopurine and thiopyrimidine drugs in experimental animals. The observation that acetylsalicylic acid and salicylic acid inhibit TPMT might have clinical importance. In man, acetylsalicylic acid is rapidly hydrolyzed to form salicylic acid (22). Therapeutic plasma concentrations of salicylic acid vary from 1 to 2 mM (23), 3–7 times the IC_{50} value for inhibition of TPMT by salicylic acid. It is possible that TPMT activity is significantly inhibited in patients receiving therapeutic doses of acetylsalicylic acid. Since salicylates are frequently used clinically, a potentially significant drug-drug interaction might occur when salicylates are administered with medications such as 6-MP. The *S*-methylation of nonheterocyclic aromatic sulfhydryl compounds by TPMT may also be of importance for human drug metabolism. For example, the widely used analgesic, acetaminophen, is excreted in part as an *S*-methyl derivative (24). The biochemical pathway responsible for the formation of the *S*-methyl metabolite is unknown. The results of the present studies raise the possibility that, in addition to its role in the metabolism of thiopurines and thiopyrimidines, TPMT might also play a role in the metabolism of even such commonly used medications as acetaminophen.

Results of the quantitative structure-activity relationship analysis have provided clues with regard to the interaction of benzoic acid inhibitors with TPMT. For example, it appeared that the hydrophobic effect played an important role for only one of the two possible *m*-substituents. This behavior is similar to the interaction of phenyl hippurate derivatives with papain (25). It is known from molecular modeling by computer graphics that the binding site of papain is a cleft with two hydrophobic regions. Only one of the two *m*-substituents can contact the hydrophobic surface. When such contact occurs, the other *m*-substituent is forced into the aqueous phase. The quantitative structure-activity relationship analysis for TPMT inhibitors raises the possibility that these compounds might interact with a similar cleft. The positive coefficient for $\sum \sigma$ shown in Eq. 2 indicated that electron-attracting substituents favor binding, and, therefore, inhibition. Future studies are required to determine whether large substituents in the *p* position can contact the enzyme, and additional 3,5-disubstituted congeners will have to be tested to establish whether only one *m*-substituent can interact with the enzyme. It should be emphasized that Eq. 2 must be viewed as preliminary in nature. Only 12 data points were used to derive this two-variable equation. In addition, there was little variation in the 4-substituents of the compounds tested, and variations in σ values were not as great as might have been desired. Since π_3' was found to be the most important variable, the role of the hydrophobic effects of 3-substituents does seem to be securely established. However, the importance of electronic effects remains to be determined.

There has been confusion with regard to the nomenclature used for enzymes that catalyze *S*-methylation. The name "thiol methyltransferase" (EC 2.1.1.9) has occasionally been applied to all *S*-methyltransferases (26). In their original report of thiol methyltransferase activity, Bremer and Greenberg (27) described a membrane-bound enzyme capable of catalyzing the *S*-methylation of "nonphysiologic" aliphatic sulfhydryl compounds. Soon afterward, Remy (1) described TPMT. He clearly differentiated this soluble cytoplasmic activity from the membrane-bound enzyme that had been described by Bremer and Greenberg (27). Recent studies performed with purified human kidney TPMT (2), human kidney microsomes (2), and human erythrocytes (4, 28) have supported the conclusion that there are two distinct *S*-methyltransferase activities in man that differ in subcellular location, substrate specificity, sensitivity to inhibitors and regulation. One of these is the microsomal thiol methyltransferase described by Bremer and Greenberg (27), and the other is TPMT. Since our experiments have shown that TPMT can catalyze the *S*-methylation of aromatic thiol compounds other than thiopurines and thiopyrimidines, we would propose that consideration be given to naming TPMT "aryl thiol methyltransferase." The microsomal activity originally described by Bremer and Greenberg (27) might then be named "alkyl thiol methyltransferase." Although there is some overlap in the substrate specificities of these two activities, the proposed nomenclature would contribute to a reduction in confusion with respect to the names of enzymes that catalyze *S*-methylation.

Finally, it is possible that TPMT may play a role in the metabolism of endogenous compounds as well as drugs. An endogenous substrate for TPMT has been reported to accumulate in the plasma of patients with chronic renal failure (29). The possible importance of this finding and the over-all role of *S*-methylation in drug metabolism have only begun to be explored. The results of the studies described here represent a significant step forward in our ability to study the regulation and possible importance of aryl thiol methyltransferase (TPMT) in the metabolism of thiopurines, thiopyrimidines, aromatic sulfhydryl compounds, and even, perhaps, of endogenous compounds.

ACKNOWLEDGMENT

We thank Luanne Wussow for her assistance with the preparation of the manuscript.

REFERENCES

1. Remy, C. N. Metabolism of thiopyrimidines and thiopurines: *S*-methylation with *S*-adenosylmethionine transmethylase and catabolism in mammalian

tissues. *J. Biol. Chem.* **239**:1078–1084 (1963).
2. Woodson, L. C., and R. M. Weinshilboum. Human kidney thiopurine methyltransferase: purification and biochemical properties. *Biochem. Pharmacol.* **32**:819–826 (1983).
3. Paterson, A. R. P., and D. M. Tidd. *6-Thiopurines, Antineoplastic and Immunosuppressive Agents*, Part II (A. C. Sartoelli and D. G. Johns, eds.). Springer-Verlag, New York, 384–403 (1975).
4. Weinshilboum, R. M., and S. L. Sladek. Mercaptopurine pharmacogenetics: monogenic inheritance of erythrocyte thiopurine methyltransferase activity. *Am. J. Hum. Genet.* **32**:651–662 (1980).
5. Woodson, L. C., J. H. Dunnette, and R. M. Weinshilboum. Pharmacogenetics of human thiopurine methyltransferase: kidney-erythrocyte correlation and immunotitration studies. *J. Pharmacol. Exp. Ther.* **222**:174–181 (1982).
6. Van Loon, J. A., and R. M. Weinshilboum. Thiopurine methyltransferase biochemical genetics: human lymphocyte activity. *Biochem. Genet.* **20**:637–658 (1982).
7. Weinshilboum, R. M. "Methylator status" and assessment of variations in drug metabolism. *Trends Pharmacol. Sci.* **1**:378–380 (1980).
8. Walker, R. C., L. C. Woodson, and R. M. Weinshilboum. Rat thiopurine methyltransferase: assay procedure, developmental changes and strain variation. *Biochem. Pharmacol.* **30**:115–121 (1981).
9. Woodson, L. C., T. P. Maus, C. Reiter, and R. M. Weinshilboum. Rat thiopurine methyltransferase: regulation by testosterone. *J. Pharmacol. Exp. Ther.* **218**:734–738 (1981).
10. Woodson, L. C., and R. M. Weinshilboum. Thiopurine methyltransferase: inhibition by benzoic acid derivatives. *Pharmacologist* **24**:127 (1982).
11. Weinshilboum, R. M., F. A. Raymond, and P. A. Pazmiño. Human erythrocyte thiopurine methyltransferase: radiochemical microassay and biochemical properties. *Clin. Chim. Acta* **85**:323–333 (1978).
12. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. *Anal. Biochem.* **72**:248–254 (1976).
13. Wilkinson, G. N. Statistical estimations in enzyme kinetics. *Biochem. J.* **80**:324–332 (1961).
14. Cleland, W. W. Computer programmes for processing enzyme kinetic data. *Nature (Lond.)* **198**:463–465 (1963).
15. Segel, I. H. *Enzyme Kinetics*. John Wiley and Sons, New York, 274–282 (1975).
16. Hansch, C. A quantitative approach to biochemical structure-activity relationships. *Acc. Chem. Res.* **2**:232–239 (1969).
17. Hansch, C. A computerized approach to quantitative biochemical structure-activity relationships. *Adv. Chem.* **114**:20–40 (1972).
18. Borchardt, R. T., J. A. Huber, and M. Housten. Catechol *O*-methyltransferase. 10. 5-Substituted 3-hydroxy-4-methoxybenzoic acids (isovanillic acids) and 5-substituted 3-hydroxy-4-methoxybenzaldehydes (isovanillins) as potential inhibitors. *J. Med. Chem.* **25**:258–263 (1982).
19. Hansch, C., and A. Leo. *Substituent Constants for Correlation Analysis in Chemistry and Biology*. Wiley-Interscience Press, New York (1979).
20. Karim, A., J. Zagarello, J. Hribar, and M. Dooley. Spironolactone. I. Disposition and metabolism. *Clin. Pharmacol. Ther.* **19**:158–169 (1976).
21. Keith, R. A., and R. M. Weinshilboum. S-Methylation of sulfhydryl drugs by human red blood cell thiol methyltransferase. *Fed. Proc.* **42**:1292 (1983).
22. Rowland, M., S. Riegelman, P. A. Harris, and S. D. Sholkoff. Absorption kinetics of aspirin in man following oral administration of an aqueous solution. *J. Pharm Sci.* **61**:379–385 (1972).
23. Koch-Weser, J. Serum drug concentrations as therapeutic guides. *N. Engl. J. Med.* **287**:227–231 (1972).
24. Hart, S. J., K. Healey, M. C. Smail, and I. C. Calder. 3-Thiomethyl-paracetamol sulfate and glucuronide: metabolites of paracetamol and N-hydroxy-paracetamol. *Xenobiotica* **12**:381–386 (1982).
25. Smith, R. N., C. Hansch, K. H. Kim, B. Omiya, G. Fukumura, C. D. Selassie, P. Y. C. Yow, J. M. Blaney, and R. Langridge. The use of crystallography, graphics, and quantitative structure-activity relationships in the analysis of the papain hydrolysis of x-phenyl hippurates. *Arch. Biochem. Biophys.* **215**:319–328 (1982).
26. Weisiger, R. A., and W. B. Jacoby. Thiol *S*-methyltransferase. *Methods Enzymol.* **77**:257–262 (1981).
27. Bremer, J., and D. M. Greenberg. Enzymatic methylation of foreign sulfhydryl compounds. *Biochim. Biophys. Acta* **46**:217–224 (1961).
28. Keith, R. A., R. T. Abrahams, P. Pazmiño, and R. M. Weinshilboum. Correlation of low and high affinity thiol methyltransferase and phenol methyltransferase activities in human erythrocyte membranes. *Clin. Chim. Acta* **131**:257–272 (1983).
29. Pazmiño, P. A., S. L. Sladek, and R. M. Weinshilboum. Thiol *S*-methylation in uremia: erythrocyte enzyme activities and plasma inhibitors. *Clin. Pharmacol. Ther.* **28**:356–367 (1980).

Send reprint requests to: Dr. Richard M. Weinshilboum, Department of Pharmacology, Mayo Clinic/Mayo Foundation, Rochester, Minn. 55905.