Thiopurine Methyltransferase: Structure-Activity Relationships for Benzoic Acid Inhibitors and Thiophenol Substrates

Matthew M. Ames,[†] Cynthia Dias Selassie,[‡] Lee C. Woodson,[§] Jon A. Van Loon,[§] Corwin Hansch,[‡] and Richard M. Weinshilboum*[§]

Division of Developmental Oncology Research and Clinical Pharmacology Unit, Mayo Clinic/Mayo Foundation, Rochester, Minnesota 55905, and Department of Chemistry, Pomona College, Claremont, California 91711. *Received March 6, 1985*

Twenty-seven substituted benzoic acids have been studied as inhibitors of partially purified human renal thiopurine methyltransferase (TPMT). Quantitative structure–activity relationship (QSAR) analysis resulted in the following equation: $pI_{50} = 1.25(\pm0.53)\pi'_3 + 0.73(\pm0.38)MR_{3,4} + 2.92(\pm0.39)$. In this equation pI_{50} is the -log of the concentration of compound that inhibits the enzyme activity by 50% (IC_{50}); π'_3 is the relative hydrophobicity of the more hydrophobic of the two meta substituents; and $MR_{3,4}$ is the molar refractivity of the more hydrophobic of the two meta substituents and of the para substituent on the phenyl ring. In addition, 14 substituted thiophenols were tested as substrates for the enzyme. All 14 thiophenols tested were excellent substrates with K_m constants (0.8–7.8 μM) that were at least 2 orders of magnitude lower than those of any known thiopurine substrate for TPMT. However, there was no discernible relationship between the activities of thiophenol substrates and their physicochemical parameters. These results suggest that benzoic acid inhibitors of and thiophenol substrates for TPMT may interact with different sites on the enzyme.

Thiopurine methyltransferase (EC 2.1.1.67, TPMT) catalyzes the S-methylation of thiopurine and thiopyrimidine drugs.[1,2] Thiol methylation is an important pathway in the metabolism of many of these drugs.[3] There are wide individual variations in human erythrocyte TPMT activity, variations that are regulated by a common genetic polymorphism.[4] Genetically determined levels of TPMT activity in the erythrocyte are correlated with individual variations of the enzyme activity in other human tissues.[5,6] Therefore, the possiblity exists that individual differences in TPMT activity might represent one factor responsible for variations in the therapeutic or toxic effects of thiopurine and thiopyrimidine drugs.[7,8] It would be useful if potent inhibitors were available for use in the study of this important drug metabolizing enzyme. It would also be important to be able to predict the relative affinities of TPMT for substrates.

We reported that benzoic acid compounds are noncompetitive inhibitors of TPMT, and that thiophenols are substrates for the enzyme.[9] Quantitative structure–activity analysis offers one approach to the design of more potent benzoic acid inhibitors. It is also one way in which a comparison can be made of the properties of the sites with which substrates and inhibitors for the enzyme interact. We have already performed preliminary quantitative structure–activity relationship studies of benzoic acid inhibitors of TPMT.[9] Equation 1 was derived as a result of those experiments. In this equation pI_{50} is the -log of

$$pI_{50} = 1.25(\pm0.64)\pi'_3 + 2.20(\pm1.24)\sum\sigma + 4.05(\pm0.32) \quad (1)$$

$$n = 12,\ r = 0.917,\ s = 0.457$$

the IC_{50} value, the concentration of compound that inhibits enzyme activity by 50%; π is a factor related to the hydrophobic or hydrophilic characteristics of substituents; σ is the normal Hammett constant; n is the number of data points used to derive the equation; r is the correlation coefficient; and s is the standard deviation. Specifically, π'_3 refers to the more hydrophobic of the two meta substituents. The less hydrophobic substituent is assumed to be oriented away from a hypothetical hydrophobic cleft in the enzyme. Therefore, π for the more polar meta substituent is assigned a value of 0. The coefficient of nearly 1 for the π term suggests complete desolvation of the type that occurs during partitioning from water to octanol.[10] That was why the hydrophobic site with which the 3-substituent interacted was visualized as a cleft rather than as a flat surface.[9] An analogous mechanism for binding has been established for the enzyme papain.[10] The σ term in eq 1 is the sum of σ values for all substituents. When independent equations were derived using π'_3 and σ, the equation in which σ was used as a variable gave results that were more poorly correlated with the observed pI_{50} values than did the equation in which π'_3 was used as a variable.[9] Finally, in our original study no parameterization was performed for 4-substituents since nearly all of the congeners tested contained the same 4-OCH_3 group.[9]

The new experiments described in the present report had three goals. First, our preliminary quantitative structure–activity experiments with benzoic acid inhibitors of TPMT were extended to include studies of 4-substituents and an attempt to test the upper limit of potency for this class of enzyme inhibitors. Second, a series of thiophenol substrates for TPMT was tested to determine whether quantitative structure–activity analysis performed

[†] Division of Developmental Oncology Research, Mayo Clinic/Mayo Foundation.
[‡] Department of Chemistry, Pomona College.
[§] Clinical Pharmacology Unit, Mayo Clinic/Mayo Foundation.
* Address all correspondence and reprint requests to: Dr. Richard Weinshilboum, Clinical Pharmacology Unit, Department of Pharmacology, Mayo Clinic/Mayo Foundation, Rochester, MN 55905.

(1) Remy, C. *J. Biol. Chem.* **1963**, *238*, 1078.
(2) Woodson, L. C.; Weinshilboum, R. M. *Biochem. Pharmacol.* **1983**, *32*, 819.
(3) Paterson, A. R. P.; Tidd, D. M. In "6-Thiopurines, Antineoplastic and Immunosuppressive Agents Part II"; Sartorelli, A. C., Johns, D. G., Ed.; Springer-Verlag: New York, 1975; pp 384–403.
(4) Weinshilboum, R. M.; Sladek, S. L. *Am. J. Human Genet.* **1980**, *32*, 651.
(5) Van Loon, J.; Weinshilboum, R. M. *Biochem. Genet.* **1982**, *20*, 637.
(6) Woodson, L. C.; Dunnette, J. H.; Weinshilboum, R. M. *J. Pharmacol. Exp. Ther.* **1982**, *222*, 174.
(7) Weinshilboum, R. M. *Trends Pharmacol. Sci.* **1980**, 378.
(8) Weinshilboum, R. M. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1984**, *43*, 2303.
(9) Woodson, L. C.; Ames, M. M.; Selassie, C. D.; Hansch, C.; Weinshilboum, R. M. *Mol. Pharmacol.* **1983**, *24*, 471.
(10) Smith, R. N.; Hansch, C.; Kim, K. H.; Omiya, B.; Fukumura, G.; Selassie, C. D.; Jow, P. Y. C.; Blaney, J. M.; Langridge, R. *Arch. Biochem. Biophys.* **1982**, *215*, 319.

0022-2623/86/1829-0354$01.50/0

Table I. Inhibition of TPMT by Benzoic Acid Derivatives[a]

compd no.[b]	substit other than hydrogen 3	4	5	pI_{50} obsd	pI_{50} calcd	Δ log pI_{50}	π'_3	$MR_{3,4}$	π_4
1		NH_2		2.54	3.39	0.85	0.00	0.64	−1.23
2*		$C(CH_3)_3$		2.68	4.44	1.76	0.00	2.06	1.98
3		OH		2.76	3.21	0.45	0.00	0.39	−0.67
4		OCH_3	OH	2.89	3.58	0.69	0.00	0.89	−0.02
5	NO_2		NO_2	2.95	3.19	0.24	−0.28	0.84	0.00
6		SO_2NH_2		3.21	3.90	0.69	0.00	1.33	−1.82
7	OH	OCH_3	OH	3.22	2.88	0.34	−0.67	1.08	−0.02
8		NO_2		3.30	3.54	0.24	0.00	0.84	−0.28
9		OH	OCH_3	3.32	3.21	0.11	0.00	0.39	−0.67
10			NO_2	3.43	3.07	0.36	0.00	0.20	0.00
11		OCH_3		3.54	3.58	0.04	0.00	0.89	−0.02
12		CH_3		3.54	3.42	0.13	0.00	0.67	0.56
13			OCH_3	3.70	3.07	0.63	0.00	0.20	0.00
14	Cl			3.71	4.33	0.62	0.71	0.70	0.00
15		Cl		3.73	3.44	0.29	0.00	0.70	0.71
16	CH_3			3.76	4.11	0.36	0.56	0.67	0.00
17	$N(CH_3)_2$	OCH_3	OH	3.90	4.13	0.23	0.18	1.34	−0.02
18		OCH_3	OCH_3	4.00	3.58	0.42	0.00	0.89	−0.02
19		I		4.12	4.02	0.10	0.00	1.49	1.12
20	CH_3		CH_3	4.48	4.12	0.36	0.56	0.67	0.00
21	Cl		Cl	4.55	4.33	0.22	0.71	0.70	0.00
22	OCH_3	OCH_3	OH	4.70	4.06	0.64	−0.02	1.58	−0.02
23*	NO_2	OCH_3	OH	5.05	3.70	1.35	−0.28	1.53	−0.02
24	Br	OCH_3	OH	5.52	5.24	0.28	0.86	1.68	−0.02
25	Cl	OCH_3	OH	5.59	4.83	0.76	0.71	1.39	−0.02
26	I	OCH_3	OH	5.92	5.93	0.01	1.12	2.18	−0.02
27	I	I	I	6.13	6.38	0.25	1.12	2.79	1.12

[a] pI_{50} values are the −log of IC_{50} values. Calculated pI_{50} values were computed by the use of eq 3 (see text for details). π'_3 is the π constant for meta substituents. π_4 is the π constant for para substituents. When there are two meta substituents, only the more hydrophobic substituent was assigned a π value. $MR_{3,4}$ is the molar refractivity of the more hydrophobic of the meta substituents and of the para substituents. [b] (*) Data from these compounds were not used in the derivation of eq 2–4. π and MR values were obtained from ref 19.

with substrates might yield information with regard to the nature of the active site of the enzyme. Finally, the characteristics of inhibitor and substrate sites were compared.

Results

The first series of experiments was designed to extend our previous structure–activity relationship studies of benzoic acid inhibitors of TPMT. A total of 27 compounds, 15 more than the number used in the preliminary study,[9] was examined. In these and in all subsequent experiments, the enzyme that was used had been purified over 300-fold from human renal tissue.[2,9] Attempts to purify the enzyme further result in its inactivation.[2] IC_{50} values for inhibition of TPMT were determined for all of the compounds studied (Table I). These values varied by almost 3 orders of magnitude. Equations 2–4 were derived by using the data shown in Table I. The abbre-

$$pI_{50} = 1.78(\pm0.57)\pi'_3 + 3.54(\pm0.28) \quad (2)$$

$$n = 25,\ r = 0.803,\ s = 0.599,\ F_{1,23} = 41.8$$

$$pI_{50} = 1.25(\pm0.53)\pi'_3 + 0.73(\pm0.38)MR_{3,4} + 2.92(\pm0.39) \quad (3)$$

$$n = 25,\ r = 0.891,\ s = 0.468,\ F_{1,22} = 15.8$$

$$pI_{50} = 1.21(\pm0.49)\pi'_3 + 0.65(\pm0.37)MR_{3,4} + 0.33(\pm0.32)\pi_4 + 3.04(\pm0.38) \quad (4)$$

$$n = 25,\ r = 0.912,\ s = 0.432,\ F_{1,21} = 4.76$$

viations and symbols used in these three equations have the same meanings as they do in eq 1. In addition, F is the Fisher statistic, and MR is the molar refractivity, a function that gives information with respect to "bulk" effects of substituents. $MR_{3,4}$ is scaled by 0.1 and refers only to the more lipophilic meta substituent and to the MR of 4-substituents. For 5-substituents, i.e., the more hydrophilic of the two meta substituents, MR was assigned a value of 0 since it was assumed that these substituents projected away from the enzyme. Each of the three equations is significant, and eq 2 and 3 are highly significant ($F_{1,23;\alpha=.001} = 14.1$; $F_{1,22;\alpha=.001} = 14.4$ and $F_{1,21;\alpha=.05} = 4.32$). Although the last term to enter eq 4, π_4, is statistically significant ($F_{1,21;\alpha=.05} = 4.32$), the small increase in the correlation coefficient for the equation suggests that π_4 is probably of relatively little importance. Because the addition of this term is of only borderline significance, and because of the wide range in the confidence limits for π_4 in eq 4 (±0.32 for a value of 0.33), in the remainder of this discussion emphasis will be placed on eq 3 rather than on eq 4. The variables in eq 2–4 are reasonably independent, as demonstrated by the data in the squared correlation matrix shown in Table II. In contrast to the results found with eq 1, no role was found for σ, the Hammett constant, when the more extensive data in Table I were used to derive the equations.

Table II. Squared Correlation Matrix for the Variables in Equations 2–4

	π'_3	π_4	$MR_{3,4}$	MR_4
π'_3	1	0.05	0.28	0.00
π_4	0.05	1	0.10	0.03
$MR_{3,4}$	0.28	0.10	1	0.57

Two of the data points in Table I, those for compounds **2** and **23** [4-$C(CH_3)_3$ and 3-NO_2, 4-OCH_3, 5-OH], were not used in the derivation of eq 2–4. Inclusion of those points would yield eq 5. The intercept and the coefficients for

$$pI_{50} = 1.26(\pm0.66)\pi'_3 + 0.64(\pm0.50)MR_{3,4} - 0.03(\pm0.41)\pi_4 + 3.01(\pm0.56) \quad (5)$$

$$n = 27,\ r = 0.795,\ s = 0.646$$

π'_3 and $MR_{3,4}$ in eq 5 are very similar to those for eq 4, but the weak π_4 term in eq 5 is not significant.

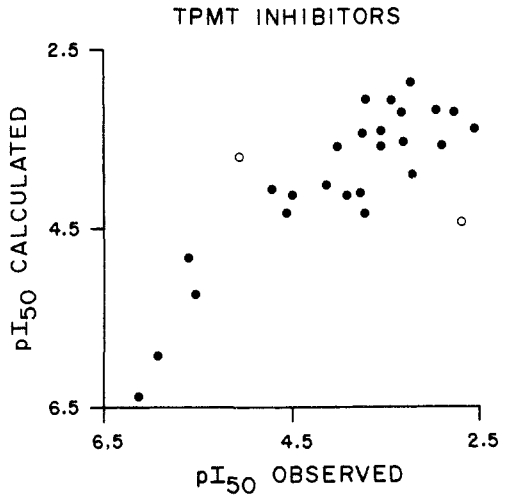

Figure 1. Correlation between experimentally observed pI_{50} values and values calculated by the use of eq 3. Data from the points shown as open circles (compounds **2** and **23** in Table I) were not used in the derivation of eq 3. See text for details.

The pI_{50} values that were measured experimentally and pI_{50} values for the same compounds calculated with eq 3 are plotted against each other in Figure 1. The correlation coefficient for the 25 points used to derive eq 3 is 0.891 ($P < 0.001$). If all 27 points are included, the correlation coefficient decreases to 0.794 ($P < 0.001$). Inspection of Figure 1 gives the impression that the correlation between experimentally observed and calculated values is especially striking for the more potent inhibitors, those with pI_{50} values of 4.5 or greater. If the two points not used in the derivation of eq 3 are neglected, the correlation coefficient for the 6 points with pI_{50} values of greater than 4.5 is 0.933 ($P < 0.01$), while that for the 19 points with pI_{50} values of less than 4.5 is 0.547 ($P < 0.05$). Therefore, even though the correlation remains significant, it is less impressive for the least potent inhibitors, those with pI_{50} values of less than 4.5.

In a final series of experiments, 14 thiophenol compounds were tested as possible substrates for TPMT. Each thiophenol tested was a substrate for the enzyme. Apparent Michaelis (K_m) constants were calculated for each compound (Table III) All 14 of these thiophenol derivatives were much better substrates than the thiopurines and thiopyrimidines previously thought to be the only substrates for the enzyme.[1,2] For example, apparent K_m constants for 6-mercaptopurine and 2-thiouracil are 550 and 2000 μM, respectively,[2] while K_m constants for all of the thiophenols that we tested were less than 10 μM. Unfortunately, unlike the IC_{50} data for benzoic acid inhibitors of TPMT (Table I), apparent K_m constants for thiophenol substrates varied by only 1 order of magnitude, from 0.8 to 7.8 μM (Table III). It was not possible to derive an equation based on their physicochemical parameters that adequately predicted the behavior of these compounds as substrates for TPMT. This difference in behavior between thiophenol substrates for and benzoic acid inhibitors of TPMT is compatible with the conclusion that they may interact with the enzyme at different sites.

Discussion

TPMT plays an important role in the metabolism of heterocyclic sulfhydryl drugs such as 6-mercaptopurine and 6-thioguanine.[1-3] We recently reported that benzoic acid derivatives are noncompetitive inhibitors of TPMT, and that TPMT catalyzes the S-methylation of nonheterocyclic thiophenol derivatives.[9] We also reported the results of preliminary structure–activity relationship experiments

Table III. Thiophenol Substrates for TPMT[a]

compd no.	compd XC_6H_5SH: substit other than hydrogen 2	3	4	apparent K_m (μM) ± SEM
1	Br			0.8 ± 0.1
2			$NHCOCH_3$	2.1 ± 0.2
3		OCH_3		2.1 ± 0.1
4		Cl		2.4 ± 0.4
5	NH_2			2.5 ± 0.2
6	OCH_3			2.6 ± 0.5
7			OCH_3	3.2 ± 0.1
8			Cl	3.6 ± 0.1
9				3.9 ± 0.5
10			Br	4.8 ± 0.3
11			F	5.6 ± 0.3
12			CH_3	5.9 ± 0.3
13			NO_2	6.5 ± 0.4
14	COOH			7.8 ± 0.8

[a] Apparent K_m constants for thiophenol and 13 thiophenol derivatives as substrates for TPMT were calculated as described in the Experimental Section. Each value is the mean ±SEM of six determinations.

with benzoic acid inhibitors—experiments intended as a first step toward the twin goals of understanding the characteristics of the site with which benzoic acid compounds interact and of designing more potent inhibitors. The results of the present series of experiments significantly extend our previous report that benzoic acid derivatives inhibit purified human kidney TPMT and that thiophenols are substrates for the enzyme.[9] Equations 2–4 reinforce our preliminary conclusion[9] that the most important factor in the binding of benzoic acid compounds to TPMT is the hydrophobicity of one meta substituent, and that the second meta substituent has relatively little effect. The apparent role for σ reported in our preliminary study[9] was most likely due to a lack of independence between the σ and π'_3 variables. Electronic factors would not be expected to significantly alter the ionization state of most benzoic acids under the pH 6.7 conditions at which the TPMT enzyme assay is performed. The lack of a significant role for σ in the present analysis suggests that other major electronic effects such as charge transfer do not influence the inhibition of TPMT by benzoic acid analogues.

The results of the present study are consistent with our original suggestion that the binding site on the enzyme for inhibitors includes a hydrophobic cleft.[9] The $MR_{3,4}$ and π'_3 variables in the equations are reasonably independent. The positive coefficient found for $MR_{3,4}$ suggests a role for relatively bulky polar groups in the activity of inhibitors. Therefore, we speculate that there may be a polar region in the vicinity of the cleft. Flexibility of this hypothetical polar region would allow for the accommodation of substituents as large as iodo without a loss of inhibitor activity. However, since compounds with a 4-*tert*-butyl group are about 50 times less active than would be expected (Table I), there would have to be an upper limit to this proposed flexibility.

Similar coefficients were obtained when MR_3 and MR_4 were modeled separately. Virtually identical results were obtained when these two variables were modeled together as a sum. The positive coefficient for MR of 4-substituents indicates that inhibition is enhanced by larger substituents, at least with groups up to the size of *tert*-butyl. Similar situations have been reported for the interaction of ligands with the cysteine hydrolases and with subtilisin.[10,11] In

(11) Carotti, A.; Casini, G.; Hansch, C. *J. Med. Chem.* 1984, *27*, 1427.

those other cases, a positive steric effect may result from the buttressing action of a polar region that helps to position the ligand in the binding site. The $MR_{3,4}$ term applies to both 3- and 4-substituents. The positive effect of 3-substituents is difficult to explain. It might be speculated that, beyond the simple partitioning modeled by π, 3-substituents also produce a distortion of the enzyme that enhances inhibition. The major effect of the weak π_4 term in eq 5 is to account for the relatively poor binding by highly polar groups such as 4-SO_2NH_2, 4-NH_2, and 4-OH. Correlations for such compounds are greatly improved by the addition of this term (data not shown). Obviously, many additional groups would have to be studied to determine the nature of the contribution to inhibition by groups in the 4-position.

As we suggested in our preliminary study,[9] one possible reason why the correlations for eq 3 and 4 are not higher might be that insufficient quantities of many of the polysubstituted derivatives were available for us to perform direct measurements of their partition coefficients. Therefore, we were forced to use π values obtained from simple monosubstituted benzenes without taking substituent interactions into account. Values for π are affected by the presence of electron-attracting groups on a benzene ring with other substituents that have lone-pair electrons.[12] For that reason, it might be expected that the most poorly predicted compounds would be those that contain the strong electron-attracting nitro group coupled with OH and OCH_3 groups. That is exactly what we found for compound **23** (Table I).

The plot of experimentally observed pI_{50} values vs. values calculated by the use of eq 3 (Figure 1) gives the impression that the predictive ability of the equation is best for the most active compounds, those with pI_{50} values of 4.5 or greater. It is unclear whether the increased "scatter" of the data for compounds with pI_{50} values of less than 4.5 is apparent or real. Fewer compounds with pI_{50} values of more than 4.5 were tested than were compounds with values less than 4.5. It is possible that the data for the two groups might appear similar if equal numbers of compounds had been tested.

After most of the compounds listed in Table I had been studied, an attempt was made to choose a very active TPMT inhibitor. We selected the commercially available 3,4,5-triiodobenzoic acid. That compound was chosen partially because of its predicted high activity as an inhibitor and partially to assess the effect on inhibition of large substituents in the 5-position. The similarity between observed and calculated pI_{50} values for this congener helped to confirm the reliability of eq 3. As more active TPMT inhibitors are sought in the future, it would be worthwhile to study compounds with more hydrophobic meta derivatives. For example, calculations performed with eq 3 yield pI_{50} values of 6.8 for 3-*tert*-butyl- and 3-*n*-butylbenzoic acids. It would be especially interesting to synthesize 4-iodo-3-*n*-butylbenzoic acid, a compound with a predicted pI_{50} value of 8. Obviously, the size of the hypothetical hydrophobic cleft with which these inhibitors interact is unknown, and it may be impossible to achieve such high levels of inhibition.

In summary, quantitative structure–activity relationship analysis has resulted in a hypothetical model for the site on TPMT with which benzoic acid inhibitors interact. That site may include a hydrophobic cleft that is large enough to accept an iodine atom attached to benzoic acid at the meta position. It may also have a flexible polar region at one end of the cleft. The accessible space between this hypothetical polar region and the other end of the cleft would have to be at least as large as 4-iodobenzoic acid, but not as large as 4-*tert*-butylbenzoic acid, a compound that is much less active as an inhibitor than predicted by eq 3. It will be of interest to determine how closely this model might resemble that which will eventually be found when X-ray crystallography of this important drug metabolizing enzyme becomes possible. Finally, the results of structure–activity relationship analysis are compatible with the conclusion that thiophenol substrates and benzoic acid inhibitors may interact with different sites on the TPMT molecule.

Experimental Section

TPMT Assay. TPMT activity was measured by the method of Weinshilboum et al., as described in detail elsewhere.[13] This assay is based on the conversion of 6-mercaptopurine (6-MP) to radioactively labeled 6-(methylthio)purine with [^{14}C-*methyl*]-*S*-adenosyl-L-methionine (Ado-Met) as the methyl donor. Substrate concentrations used in the assay were 3.7 mM for 6-MP and 25 μM for Ado-Met. The pH of the reaction was 6.7. After incubation at 37 °C for 30 min, the enzyme reaction was terminated by the addition of 0.5 M borate buffer, pH 10. 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. One unit of TPMT activity represented the formation of 1 nmol of 6-(methylthio)purine/h of incubation at 37 °C.

TPMT Inhibitor Studies. The ability of a series of benzoic acid compounds to inhibit TPMT was studied. The purity of the inhibitors was determined by high-performance liquid chromatography performed with an IBM Model 9533 pump equipped with an IBM minicomputer and an IBM autosampler with a 10-μm C_{18} reversed-phase IBM column. The elution solvent was 0.1 M sodium dihydrogen phosphate buffer in methanol. The pH of the buffer was varied from 4.2 to 4.5, and the solvent system composition was varied from 50:50 (buffer/methanol) to 75:25, depending on the individual compound studied. Detection was by UV absorption at 254 nm. With the exception of 3,4,5-triiodobenzoic acid, all compounds eluted as a single peak. Commercially available 3,4,5-triiodobenzoic acid contained a secondary peak that made up 25% of the total peak area. Therefore, this compound was purified by recrystallization from methanol, recrystallization from tetrahydrofuran/hexane (80:20, v/v), and preparative thin-layer chromatography (indicating silica plates, tetrahydrofuran/ethyl acetate (20:80, v/v) solvent system). The desired product (R_f 0.70) was eluted with tetrahydrofuran. The structure and purity of the recrystallized compound were verified by mass spectrometric and NMR analysis and by high-performance liquid chromatography. For enzyme inhibition studies, each potential inhibitor was dissolved in Me_2SO for use in the enzyme assay. In all cases, control samples that contained only Me_2SO were also tested. The enzyme assay was performed exactly as it was for the measurement of TPMT activity. Initially, the effects of at least five different concentrations of inhibitor ranging from 10^{-2} to 10^{-6} M were studied. Once an appropriate concentration range had been determined, a series of concentrations including at least four near that required to inhibit enzyme activity by 50%, the IC_{50} value, were used. 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. The values shown in Table I are averages of the two independent determinations of IC_{50} values.

TPMT Substrate Studies. A series of aromatic sulfhydryl compounds were tested as possible TPMT substrates. These assays were identical with the standard enzyme assay except that 6-MP was replaced with the compound to be tested. The purity

(12) Leo, A. *J. Chem. Soc., Perkin Trans. 2* **1983**, *2*, 825.

(13) Weinshilboum, R. M.; Raymond, F. A.; Pazmino, P. A. *Clin. Chim. Acta* **1978**, *62*, 248.

of each sulfhydryl compound was determined by high-performance liquid chromatography performed with a 10-μm C_{18} reversed-phase IBM column with water/methanol (50:50) as the solvent and with detection by UV absorption at 254 nm. All of the compounds eluted as single peaks. Initial experiments with each compound were performed with a series of concentrations that differed by several orders of magnitude. Apparent K_m constants were then estimated on the basis of data from experiments performed with at least five different concentrations close to the K_m constant for that compound. The effect of each concentration was measured in triplicate, and each experiment was performed a minimum of two times. The final apparent K_m constants reported are the averages of all values determined.

Protein Assay. Protein concentrations were measured by the dye binding method of Bradford[14] with bovine serum albumin as a standard.

Purification of TPMT. Human kidney TPMT was purified as described elsewhere.[2] Briefly, human renal tissue was obtained from patients undergoing clinically indicated nephrectomies. The kidneys were obtained under guidelines established by the Mayo Clinic Institutional Review Board. The renal tissue was homogenized, and a supernatant was obtained after centrifugation at 100000*g* for 1 h. The supernatant was subjected to ammonium sulfate precipitation, ion-exchange chromatography performed 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 in the supernatant after centrifugation at 100000*g*. Other characteristics of the purified enzyme have been described elsewhere.[2]

Kinetic Analyses. Michaelis (K_m) constants were estimated by the method of Wilkinson[15] with a computer program written by Cleland.[16] A Hewlett Packard Model 9845B computer was used to perform these calculations.

Quantitative Structure–Activity Relationship Analysis. Quantitative structure–activity relationship analysis was performed by the method of Hansch.[17,18]

Materials. [^{14}C-*methyl*]-Ado-Met (sp. act. 58 mCi/mmol) was purchased from New England Nuclear Corp., Boston, MA. Ado-Met·HCl, allopurinol, crystalline bovine serum albumin, dithiothreitol, vanillic acid, *p*-hydroxybenzoic acid, thiophenol, DEAE-A25 ion-exchange resin, and Sephadex G-100 were purchased from Sigma Chemical Co., St. Louis, MO. Veratric acid, 3,5-dimethylbenzoic acid, 3-nitrobenzoic acid, 3- and 4-anisic acid, 3- and 4-toluic acid, 3- and 4-chlorobenzoic acid, 2- and 4-bromothiophenol, 3- and 4-chlorothiophenol, 4-fluorothiophenol, 4-nitrothiophenol, 4-acetamidothiophenol, 4-thiocresol, and thiosalicylic acid were purchased from Aldrich Chemical Co., Milwaukee, WI. 2-Mercaptoaniline and 2-, 3-, and 4-mercaptoanisole were purchased from Pfaltz and Bauer Inc., Stanford, CT. 3,4-Dimethoxy-5-hydroxybenzoic acid, 3,5-dinitrobenzoic acid, and 4-*tert*-butylbenzoic acid were purchased from ICN Pharmaceuticals, Plainview, NY. *p*-Iodobenzoic acid was purchased from Eastman Kodak Co., Rochester, NY. 3,4,5-Triiodobenzoic acid was purchased from Chemicals Procurement Laboratories Inc., College Point, NY. Dye reagent for the protein assay was purchased from Bio-Rad Laboratories, Richmond, CA. Several other benzoic acid derivatives were donated by Dr. R. T. Borchardt, The University of Kansas, Lawrence, KS. These compounds have been listed elsewhere.[9]

Acknowledgment. Supported in part by National Institutes of Health Grants GM 28157 (R.M.W.), CMC-30302 (C.H.), HL 07269 (L.C.W. and R.M.W.), and RCDA CA 00755 (M.M.A.). Dr. Weinshilboum is a Burroughs Wellcome Scholar in Clinical Pharmacology.

Registry No. TPMT, 67339-09-7; 4-$H_2NC_6H_4CO_2H$, 150-13-0; 4-*t*-$BuC_6H_4CO_2H$, 98-73-7; 4-$HOC_6H_4CO_2H$, 99-96-7; 4-$H_2NSO_2C_6H_4CO_2H$, 138-41-0; 4-$O_2NC_6H_4CO_2H$, 62-23-7; 3-$O_2NC_6H_4CO_2H$, 121-92-6; 4-$MeOC_6H_4CO_2H$, 100-09-4; 4-$MeC_6H_4CO_2H$, 99-94-5; 3-$MeOC_6H_4CO_2H$, 586-38-9; 3-$ClC_6H_4CO_2H$, 535-80-8; 4-$ClC_6H_4CO_2H$, 74-11-3; 3-$MeC_6H_4CO_2H$, 99-04-7; 4-$IC_6H_4CO_2H$, 619-58-9; 2-BrC_6H_4SH, 6320-02-1; 4-$AcNHC_6H_4SH$, 1126-81-4; 3-$MeOC_6H_4SH$, 15570-12-4; 3-ClC_6H_4SH, 2037-31-2; 2-$H_2NC_6H_4SH$, 137-07-5; 2-$MeOC_6H_4SH$, 7217-59-6; 4-$MeOC_6H_4SH$, 696-63-9; 4-ClC_6H_4SH, 106-54-7; PhSH, 108-98-5; 4-BrC_6H_4SH, 106-53-6; 4-FC_6H_4SH, 371-42-6; 4-MeC_6H_4SH, 106-45-6; 4-$O_2NC_6H_4SH$, 1849-36-1; 4-$HO_2CC_6H_4SH$, 147-93-3; 3-hydroxy-4-methoxybenzoic acid, 645-08-9; 3,5-dinitrobenzoic acid, 99-34-3; 3,5-dihydroxy-4-methoxybenzoic acid, 4319-02-2; 4-hydroxy-3-methoxybenzoic acid, 121-34-6; 3-hydroxy-4-methoxy-5-(dimethylamino)benzoic acid, 80547-67-7; 3,4-dimethoxybenzoic acid, 93-07-2; 3,5-dimethylbenzoic acid, 499-06-9; 3,5-dichlorobenzoic acid, 51-36-5; 3-hydroxy-4,5-dimethoxybenzoic acid, 1916-08-1; 3-hydroxy-4-methoxy-5-nitrobenzoic acid, 80547-65-5; 3-bromo-5-hydroxy-4-methoxybenzoic acid, 52783-66-1; 3-chloro-5-hydroxy-4-methoxybenzoic acid, 80547-63-3; 3-hydroxy-5-iodo-4-methoxybenzoic acid, 80547-64-4; 3,4,5-triiodobenzoic acid, 2338-20-7.

(14) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
(15) Wilkinson, G. N. *Biochem. J.* **1961**, *80*, 324.
(16) Cleland, W. W. *Nature (London)* **1983**, *198*, 463.
(17) Hansch, C. *Acc. Chem. Res.* **1969**, *2*, 232.
(18) Hansch, C. *Adv. Chem.* **1972**, *114*, 20.
(19) Hansch, C.; Leo, A. In "Substituent Constants for Correlation Analysis in Chemistry and Biology"; Wiley: New York, 1979.