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# Sulphasalazine inhibition of thiopurine methyltransferase: possible mechanism for interaction with 6-mercaptopurine and azathioprine

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Thiopurine drugs are used in the treatment of inflammatory bowel disease—as are sulphasalazine and its metabolite 5-aminosalicylic acid (ASA). S-Methylation catalyzed by thiopurine methyltransferase (TPMT) is a major pathway in the metabolism of thiopurines. The hypothesis was tested that TPMT might be inhibited by sulphasalazine or isomers of ASA. Sulphasalazine as well as 3-, 4- and 5-ASA inhibited recombinant human TPMT, with $IC_{50}$ values of 78, 99, 2600 and 1240 μM, respectively. Kinetic studies demonstrated that the inhibition of TPMT by sulphasalazine and ASA isomers was non-competitive with regard to the thiopurine substrate, 6-MP, and was uncompetitive with regard to the methyl donor for the reaction, S-adenosyl-L-methionine. Our observations raise the possibility of a clinically significant drug–drug interaction in patients treated simultaneously with sulphasalazine and thiopurine drugs.

**Keywords** thiopurine methyltransferase sulphasalazine aminosalicylic acid 6-mercaptopurine azathioprine

## Introduction

6-Mercaptopurine (6-MP) and azathioprine are thiopurine drugs that are used in the therapy of inflammatory bowel disease [1–4]. Unfortunately, the therapeutic index of these drugs is relatively narrow [5], and they are capable of causing toxic side effects such as myelosuppression [5, 6]. For example, in one large clinical study of inflammatory bowel disease, thiopurine drugs had to be withdrawn in 10% of patients because of the occurrence of adverse reactions [1]. Over half of the patients included in that study had been treated simultaneously with 6-MP and sulphasalazine [1]. Thiopurines are metabolized by S-methylation catalyzed by the enzyme thiopurine methyltransferase (TPMT, EC 2.1.1.67) and oxidation catalyzed by xanthine oxidase (EC 1.2.3.2) [5, 7, 8]. Levels of TPMT activity in human tissues are controlled by a genetic polymorphism, with allele frequencies such that approximately 89% of the population is homozygous for high enzyme activity, approximately 11% is heterozygous and has intermediate activity, and one of every 300 subjects is homozygous for low TPMT activity in all tissues that have been studied [9–11]. Individuals with genetically low TPMT activity are at greatly increased risk for the occurrence of potentially life-threatening thiopurine toxicity such as myelosuppression when treated with standard doses of these drugs [12–14]. It has been reported that TPMT can be inhibited by derivatives of benzoic acid [15]. Since sulphasalazine and its metabolite 5-aminosalicylic acid (5-ASA) are derivatives of benzoic acid, the possibility exists that patients treated simultaneously with these agents and with thiopurines might be at risk for inhibition of an important metabolic pathway for 6-MP and azathioprine. We tested the hypothesis that sulphasalazine and/or isomers of ASA might inhibit TPMT. Both sulphasalazine and 5-ASA were found to be inhibitors of the enzyme, raising the possibility of a clinically significant drug–drug interaction in patients treated simultaneously with these agents and with thiopurines.

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## Methods

### *TPMT assay*

TPMT activity was measured with a modification of the method of Weinshilboum *et al.* [11, 16]. This enzyme assay is based on the conversion of 6-MP to radioactively labelled 6-methylmercaptopurine with [$^{14}$C]-methyl-S-adenosyl-L-methionine ([$^{14}$C]-methyl-Ado-Met, 24 μCi μmol$^{-1}$) as the methyl donor. The final concentrations of 6-MP and Ado-Met present in the reaction mixture were 7.5 mM and 24.2 μM, respectively. Blank samples contained no 6-MP. The 6-methylmercaptopurine formed during the reaction was separated from radioactively labelled Ado-Met by organic solvent extraction, and the radioactivity of the organic solvent phase was measured in a liquid scintillation counter. Each sample was assayed in duplicate, and results reported are averages of those two determinations. The interassay coefficient of variation for the TPMT assay is 4.1% [11].

### *Recombinant human TPMT*

The recombinant human TPMT used in these experiments was present in a 100,000 *g* supernatant preparation obtained from COS-1 cells that had been transfected with the eukaryotic expression vector p91023(B) which contained the 5'-untranslated region, the coding region and a portion of the 3'-untranslated region of human T84 colon carcinoma cell TPMT cDNA [17]. Detailed descriptions of the transfection of COS-1 cells as well as characteristics of the recombinant enzyme have been published elsewhere [17]. The specific activity of this enzyme preparation was 535 nmol h$^{-1}$ mg$^{-1}$ protein, and approximately 0.4 μg protein was present in each assay. The biochemical and physical properties of the COS-1 cell expressed enzyme that have been studied are identical with those of TPMT in normal human tissues [17].

### *Inhibition kinetic experiments*

Compounds tested as potential TPMT inhibitors were dissolved in dimethylsulphoxide and were added to the enzyme reaction mixture in 10 μl aliquots. Control samples to which only 10 μl dimethylsulphoxide had been added were also assayed. Initial experiments involved concentrations of inhibitor that varied by several orders of magnitude. Subsequently, a series of concentrations at or near those required to inhibit the enzyme activity by 50% were studied for each compound. $IC_{50}$ values were estimated from semilogarithmic plots of concentration-effect curves by use of the GraphPAD InPlot curve-fitting program (GraphPAD Software, San Diego, CA). $K_{ii}$ and $K_{is}$ values were determined from replots of data obtained by assaying TPMT activity in the presence of varying concentrations of the inhibitor and varying concentrations of the substrates for the reaction, either 6-MP (six concentrations ranging from 0.23 to 7.5 mM) or Ado-Met (five concentrations ranging from 1.51 to 24.2 μM). Those data were used to construct double inverse plots using the method of Wilkinson [18] with a computer progam written by Cleland [19]. $K_{ii}$ and $K_{is}$ values were then calculated as described by Segel [20]. $K_{is}$ is a measure of the effect of an inhibitor on the slope of a double inverse plot, while $K_{ii}$ is a measure of the effect of an inhibitor on the intercept of such a plot.

### *Materials*

[$^{14}$C]-Methyl-Ado-Met (60 mCi mmol$^{-1}$) was purchased from Dupont-NEN, Boston, MA. Ado-Met HCl, dimethylsulphoxide, 6-MP, 3-, 4-, and 5-ASA, sulphasalazine, sulphapyridine and pyridine were obtained from Sigma Chemical Company, St Louis, MO. 3,4-Dimethoxy-5-hydroxybenzoic acid (DMHBA) was purchased from ICN Pharmaceuticals, Plainview, NY.

## Results

The first experiment was designed to determine whether sulphasalazine, sulphapyridine, pyridine or three isomers of ASA might be capable of inhibiting the activity of TPMT. A series of concentrations of each compound was tested under optimal conditions for the assay of TPMT activity with 6-MP as the methyl acceptor substrate. The potent and well-characterized benzoic acid inhibitor of TPMT, DMHBA [15], was also tested as a control. The $IC_{50}$ value for DMHBA was 14 μM (Figure 1). Sulphasalazine, as well as 3-, 4-, and 5-ASA, also inhibited TPMT (Figure 1). However, neither sulphapyridine, another metabolite of sulphasalazine, nor pyridine displayed detectable inhibition at the highest concentration tested (1 mM). Other than DMHBA, sulphasalazine and 3-ASA were the most potent inhibitors of the compounds studied, with $IC_{50}$ values of 78 and 99 μM, respectively. $IC_{50}$ values for 4- and 5-ASA were much higher, at 2600 and 1240 μM, respectively. To determine whether inhibition of the enzyme by sulphasalazine and ASA isomers might have resulted from interference in the organic solvent extraction

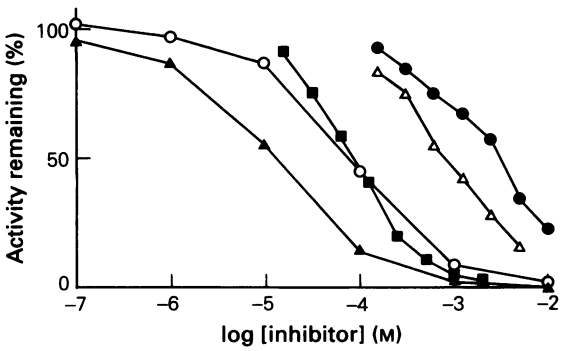


**Figure 1** TPMT inhibition by sulphasalazine (○), 3- (■), 4- (●), and 5-ASA (△) and by a known inhibitor of the enzyme, 3,4-dimethoxy-5-hydroxybenzoic acid (DMHBA, ▲). Each point is the average of two determinations.

**Table 1** Inhibition constants for inhibition of TPMT by sulphasalazine and ASA isomers. No $K_{is}$ values are listed for inhibition *vs* Ado-Met, since that inhibition was uncompetitive; i.e. the inhibitor had no effect on slope values

| | Substrate | | |
|---|---|---|---|
| | 6-MP | | Ado-Met |
| *Inhibitor* | $K_{is}$ (μM) | $K_{ii}$ (μM) | $K_{ii}$ (μM) |
| Sulphasalazine | 43 | 141 | 81 |
| 5-ASA | 1260 | 979 | 986 |
| 4-ASA | 4640 | 1510 | 2900 |

step used in the enzyme assay, the highest concentration of each compound used to obtain the data shown in Figure 1 was added to the reaction mixture after termination of the enzyme reaction but prior to the organic solvent extraction. No inhibition occurred, indicating that sulphasalazine and the ASA isomers had to be present during the enzyme reaction to inhibit TPMT, i.e. that inhibition was not an artifact of the assay procedure and did not result from alteration of the partition coefficient of the product of the enzyme reaction by the inhibitors. The next step was to perform inhibition kinetic experiments to characterize the nature of the inhibition of TPMT by these compounds.

Inhibition kinetic studies were performed with sulphasalazine and with 4-ASA and 5-ASA since those compounds are used clinically. The effect of each inhibitor was studied in the presence of a series of concentrations of both 6-MP and Ado-Met (see Methods). Each of the drugs was found to be a non-competitive inhibitor of TPMT with respect to 6-MP and an uncompetitive inhibitor with respect to Ado-Met, as reported previously for DMHBA and other benzoic acid derivatives [15]. The $K_{ii}$ and $K_{is}$ values for the inhibition of recombinant human TPMT by sulphasalazine, 4-ASA and 5-ASA listed in Table 1 demonstrate that sulphasalazine was the most potent inhibitor of these three compounds, followed by 5- and 4-ASA.

## Discussion

Thiopurine drugs are used to treat inflammatory bowel disease [1–4]. Clinical studies of patients with neoplastic disease and transplantation recipients have demonstrated that the genetic polymorphism regulating TPMT activity in humans is an important factor responsible for individual variation in the occurrence of thiopurine-induced toxicity [12–14, 21, 22]. Therefore, this genetic polymorphism almost certainly also represents a risk factor for thiopurine toxicity in patients with inflammatory bowel disease who are treated with these agents. However, these patients may be at additional risk as a result of the administration of other drugs that are used to treat their illness. It was reported previously that benzoic acid derivatives are capable of inhibiting TPMT [15], although, prior to the present study, ASA derivatives had not been tested as inhibitors of the enzyme. Those observations raised the possibility that sulphasalazine and ASA isomers such as the sulphasalazine metabolite, 5-ASA, might also inhibit the enzyme.

Sulphasalazine and all three positional isomers of ASA were able to inhibit TPMT—with sulphasalazine being the most potent inhibitor of the compounds tested (Figure 1). When sulphasalazine is administered orally, from 15–30% of the drug is absorbed in the small intestine, resulting in plasma sulphasalazine concentrations of 10–20 μg $ml^{-1}$ after a single 2 g oral dose [23, 24]. Those values correspond to serum concentrations of 25–50 μM, close to the $IC_{50}$ and $K_{ii}$ values for the inhibition of TPMT. The diazo bond that joins the sulphapyridine and 5-ASA portions of the sulphasalazine molecule is reduced by bowel flora in the colon to release these two compounds *in vivo* [25]. Since sulphasalazine is administered in relatively high doses, is significantly absorbed in the gut and is a potent inhibitor of TPMT, our *in vitro* studies raise the possibility of a clinically significant drug–drug interaction in patients treated simultaneously with sulphasalazine and thiopurines. At the very least, caution should be exercised in the use of these drugs in combination. This would be especially true in the 11% of patients who are heterozygous for the TPMT genetic polymorphism, patients who already have a reduction of enzyme activities to values only approximately half of those present in tissues of the majority of patients [9]. Future studies will be required to determine the possible clinical significance of the observation that sulphasalazine and ASA isomers are inhibitors of a major pathway in thiopurine drug metabolism.

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