Short Communication

Inhibition of Human Thiopurine S-Methyltransferase by Various Nonsteroidal Anti-inflammatory Drugs in Vitro: A Mechanism for Possible Drug Interactions

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ABSTRACT:

Thiopurine S-methyltransferase (TPMT) is a biotransformation phase II enzyme responsible for the metabolic inactivation of thiopurine drugs. The present study was carried out to investigate the inhibitory potential of 15 nonsteroidal anti-inflammatory drugs (NSAIDs) on human TPMT activity in vitro. TPMT activity was measured in pooled human erythrocytes in the absence and presence of various NSAIDs using the previously published high-performance liquid chromatography–UV method. To determine the inhibition type and K_i value for each compound, we performed kinetic analysis at five different inhibitor concentrations close to the IC_{50} value obtained in preliminary experiments. Naproxen (K_i = 52 μM), mefenamic acid (K_i = 39 μM), and tolfenamic acid (K_i = 50 μM) inhibited TPMT activity in a noncompetitive manner. The estimated K_i values for the inhibition of TPMT by ketoprofen (K_i = 172 μM) and ibuprofen (K_i = 1043 μM) indicated that the propionic acid derivatives were relatively weak inhibitors of TPMT. Our results suggest that coadministration of thiopurines and various NSAIDs may lead to drug interactions.

In addition to benzoic acid derivatives, clinically significant interactions via TPMT inhibition by furosemide, bendroflumethiazide, and trichlormethiazide may occur when administered simultaneously with thiopurines (Lysaa et al., 1996). Drugs such as prednisone, prednisolone, 6-methylprednisolone, cyclophosphamide, methotrexate, and trimethoprim–sulfamethoxazole, often used simultaneously with thiopurines in leukemic patients, had no effect on TPMT activity in vitro using lysates of red blood cells (RBC) (Jacqz-Aigrain et al., 1994).

Nonsteroidal anti-inflammatory drugs (NSAIDs) exhibit pharmacological effects similar to those of aminosalicylates. These agents also have common features in their chemical structure. We hypothesized that structural determinants responsible for the pharmacological action of NSAIDs might be involved in the inhibition of TPMT in a manner similar to aminosalicylates and benzoic acid derivatives. The potential for diclofenac, lornoxicam, piroxicam, meloxicam, ibuprofen, ketoprofen, flurbiprofen, naproxen, celecoxib, acetylsalicylic acid, mefenamic acid, tolfenamic acid, metilazole, paracetamol, and nabumetone to inhibit TPMT activity and to cause drug interactions with thiopurines was studied in vitro. Olsalazine and allopurinol were used as a positive and negative control, respectively. The concentration required to inhibit TPMT activity by 50% (IC_{50}), inhibition type, and inhibition constant (K_i) were determined for each compound.

Materials and Methods

Chemicals. Mefenamic acid, nabumetone, meloxicam sodium, tolfenamic acid, flurbiprofen, and naproxen were obtained from Sigma Aldrich (Steinheim, Germany). Allopurinol and lornoxicam were kindly provided by Nycomed (Roskilde, Denmark), olsalazine, celecoxib, and diclofenac by Pfizer Inc. (New York, NY), and acetylsalicylic acid, metamizole sodium, piroxicam,
paracetamol, ibuprofen, and ketoprofen by Tallinna Pharmaceutical Company (Tallinn, Estonia). Olsalazine, lornoxicam, piroxicam, and acetylsalicylic acid were dissolved in water. All other compounds were dissolved in DMSO, which did not exceed 2.5% (v/v) in the reaction mixture.

**TPMT Activity Assay.** In brief, erythrocyte TPMT activity was determined in vitro using 6-MP as a substrate (Oselin et al., 2006). Samples were incubated at 37°C for 60 min in a total volume of 410 μL. Samples consisted of 125 μL of 0.1 M NaH2PO4 buffer (pH = 7.4), 50 μL of 6-MP (in a final concentration of 1.0 mM), 25 μL of S-adenosyl-L-methionine (40 μM), and dithiothreitol (1 mM) blend. Depending on the compound under investigation, 10 μL of solution in water or DMSO was added. Reactions were started by adding 200 μL of hemolysate. Samples were extracted with acetonitrile, and 40 μL of extracted sample was injected into the high-performance liquid chromatography system and analyzed at a flow rate of 1.3 ml/min in an isocratic elution with a mobile phase of 0.04 M phosphate buffer and methanol (80:20 v/v), pH 7.9. For the 6-MP metabolite, 6-methylmercaptopurine (6-MMP), absorbance was detected at 290 nm. TPMT activity was expressed as the formation of 6-MMP (ng/ml) after 60 min of incubation at 37°C.

**TPMT Inhibition Studies.** TPMT inhibition studies were carried out using human erythrocytes as a source of enzyme. Hemolysates from four healthy individuals were prepared as described previously (Oselin et al., 2006), pooled, and stored at −80°C until used throughout all experiments. TPMT activity in pooled hemolysates was 118 ng/ml 6-MMP formation per 60-min incubation, which indicates normal wild-type TPMT (Oselin et al., 2006). Potential inhibitors were added to the reaction mixture in various concentrations in a 10-μl solution in water or DMSO. Control samples (without inhibitor) included 10 μl of water or DMSO, but no inhibitors. TPMT activity measurements were performed as described above. TPMT activity in the control sample was set at 100%.

To determine the concentration required to inhibit TPMT activity by 50% (IC50), initial experiments were performed at 0, 1, 10, 50, 100, and 1000 μM concentration of each compound. If no TPMT activity inhibition at the highest concentration was observed, inhibitors were further studied at concentrations of 0, 500, 1000, 2000, and 4000 μM. The TPMT substrate, 6-MP, concentration was 1.0 mM. All experiments were performed as duplicate experiments. To find the inhibition type and inhibition constant, Ki, each compound was studied at five different inhibitor concentrations close to the IC50 value and at various concentrations (0–1.2 mM) of substrate in duplicates.

**Data Analysis.** IC50 values were calculated using the WinNonlin inhibitory Imax ordinary or sigmoid library model (WinNonlin 5.0; Pharsight Corporation, Mountain View, CA). The goodness of fit of the model was determined based on the precision of model estimates, Akaike Information Criteria, and weighted residuals sum of squares. For the ordinary Imax model, IC50 was calculated from the equation enzyme activity (V) = Imax \times \frac{1}{1 + [I/(IC_{50})]}, where I is the concentration of inhibitor. For the sigmoid Imax model, IC50 was calculated from the equation V = Imax \times \frac{1}{1 + ([I/(IC_{50})]^n)}, where n is a sigmoidicity factor.

The inhibition mechanism, namely, competitive, uncompetitive, noncompetitive, or mixed, was observed by visual inspection of graphical plots after data linearization (Lineweaver-Burk plot) and by using the GraphPad Prism software (ver 4.0; GraphPad Software Inc., San Diego, CA). For all compounds the noncompetitive inhibition model gave the best fit to the data. The inhibitory constant, Ki, was estimated by simultaneous nonlinear regression, and enzyme activity, V, was calculated from the following equation: V = \frac{[V_{\text{max}} \times C/(K_C + C) \times \gamma K/K_s + I]}{I + [IC_{50} + C]}, where C is the concentration of substrate and I is the concentration of inhibitor (WinNonlin 5.0; Pharsight Corporation).

### Results and Discussion

**Determination of IC50 Value.** For naproxen, tolenamic acid, and mefenamic acid, the sigmoid Imax inhibitory effect model captured the curvature in the data better and was used to calculate the IC50 value (Table 1). For all other compounds the best fit was obtained using the ordinary Imax inhibitory effect model. Allopurinol, acetylsalicylic acid, and metamizole did not show TPMT inhibitory activity at the highest concentration tested (residual TPMT activity >90%), and no Ki determinations were performed. For all other compounds, inhibition type and Ki value were determined at five different inhibitor concentrations selected close to the IC50 value as obtained from initial experiments.

**Inhibition Kinetics.** Figure 1 plots TPMT activity determined in the presence of the most potent inhibitors, naproxen, mefenamic acid, and tolenamic acid. The Ki values for naproxen (Ki = 52 μM), mefenamic acid (Ki = 39 μM), and tolenamic acid (Ki = 50 μM) were very close to the IC50 values obtained in initial experiments, which confirms noncompetitive inhibition (Vmax decreased, but no effect on IC50) (Table 1). Ibuprofen and ketoprofen inhibited TPMT with Ki values of 1043 μM and 172 μM, respectively. No drug interactions between thiopurines and other compounds tested are expected in clinical practice (Table 1).

The current study was carried out to investigate the inhibitory potential of 15 different NSAIDs on human TPMT activity using RBC as a source of enzyme. Naproxen, tolenamic acid, and mefenamic acid were found to inhibit TPMT with Ki values lower than or comparable to serum concentrations in patients on respective therapy. In certain circumstances, also, ibuprofen and ketoprofen might have potential to inhibit TPMT activity in a clinically significant manner. Diclofenac, flurbiprofen, lornoxicam, celecoxib, piroxicam, paracetamol, nabumetone, and meloxicam inhibited TPMT in concentrations higher than those that have been determined in human serum. All potential inhibitors were noncompetitive inhibitors of TPMT, indicating that TPMT inhibition occurs via binding to a site different from substrate binding site. The noncompetitive type of inhibition kinetics has been shown for other compounds known to inhibit TPMT.

In the area of pharmacogenetics, TPMT is one of the best studied targets. TPMT pheno- or genotyping before initiation of thiopurine therapy and dose reduction of 50% to 90% in individuals with intermediate and low TPMT activity is recommended. Thiopurines 6-MP, 6-TG, and azathioprine are prodrugs with no pharmacological effect. Intracellular formation of thioguanine nucleotides via the hypoxanthine phosphoribosyltransferase has a major role in the efficacy and toxicity of thiopurines (Lennard et al., 1983). Alternatively, metabolic conversion of thiopurines via TPMT or xanthine oxidase leads to the formation of inactive methylated and oxidized metabolites, respectively. Inhibition of xanthine oxidase by allopurinol results in excess formation of thioguanine nucleotides and an increased incidence of side effects of thiopurines when coadministered with allopurinol. The latter is a well known drug interaction.

Unknown drug interactions are often difficult to recognize. Drug interactions with thiopurines may lead to increased toxicity and cessation of thiopurine therapy due to misinterpretation of toxic effects as

<table>
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<tr>
<th>Compound</th>
<th>IC50 Value</th>
<th>K_i Value</th>
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<tr>
<td>Mefenamic acid</td>
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<td>39</td>
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<td>Naproxen</td>
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**TABLE 1** The IC50 and K_i values for the in vitro inhibition of human TPMT by compounds tested.
Experiments were performed as duplicates. Simultaneous nonlinear regression (WinNonlin 5.0.1, Pharsight Corporation) was used to determine IC50 values for sulfasalazine, olsalazine, and mesalazine, respectively, using the noncompetitive inhibition type and concentrations 0, 12.5 µM, 25 µM, 50 µM, and 100 µM. Ki values of 52 µM, 39 µM, and 50 µM were calculated for naproxen, mefenamic acid, and tolfenamic acid, respectively, using the noncompetitive inhibition type and simultaneous nonlinear regression (WinNonlin 5.0.1, Pharsight Corporation). Experiments were performed as duplicates.

None of the previous studies which aimed to study olsalazine potential to inhibit TPMT have used RBC as a source of enzyme. A 10-fold difference in IC50 values has been reported for sulfasalazine, depending on whether the recombinant enzyme [IC50 = 70–78 µM (Szumlanski and Weinshilboum, 1995; Lewis et al., 1997)] or RBC [IC50 = 640 µM (Shipkova et al., 2004)] were used as a source of enzyme. Similar findings have been reported for diuretics (Lysaa et al., 1996; Xin et al., 2005). The magnitude of inhibition observed in vitro experiments may depend on the source of enzyme and on experimental conditions. This should be taken into account when interpreting results from the present study.

In conclusion, we found that naproxen, tolfenamic acid, and mefenamic acid inhibited human TPMT in vitro, and clinically significant drug interactions may occur when thiouropines are used simultaneously with various NSAIDs. Further in vivo drug interaction studies are needed to confirm the clinical relevance of the present finding.

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