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# Inhibition of Human Thiopurine S-Methyltransferase (TPMT) by Various NSAIDs in vitro: a Mechanism for Possible Drug Interactions

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**Running title:** TPMT inhibition by NSAIDs

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**List of abbreviations:**

TPMT, thiopurine S-methyltransferase; RBC, red blood cells; 6-MP, 6-mercaptopurine; 6-MMP, 6-methylmercaptopurine; DMSO, dimethylsulfoxide; DTT, dithiothreitol; IC50, inhibitor concentration required to inhibit enzyme activity by 50%; V, enzyme activity; Imax, maximum inhibition effect.

## 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 non-steroidal 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 HPLC-UV method. To determine the inhibition type and Ki value for each compound we performed kinetic analysis at five different inhibitor concentrations close to the IC50 value obtained in preliminary experiments. Naproxen (Ki=52 μM), mefenamic (Ki=39 μM) and tolfenamic acid (Ki=50 μM) inhibited TPMT activity in a noncompetitive manner. The estimated Ki values for the inhibition of TPMT by ketoprofen (Ki=172 μM) and ibuprofen (Ki=1043 μM) indicated that the propionic acid derivatives were relatively weak inhibitors of TPMT. Our results suggest that co-administration of thiopurines and various NSAIDs may lead to drug interactions.

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Thiopurine S-methyltransferase (TPMT, EC 2.1.1.67) is a biotransformation phase II enzyme involved in inactivation of the thiopurine drugs azathioprine, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) (Anglicheau et al., 2002). Individuals with genetically determined low or intermediate TPMT activity have a higher risk for side effects when treated with standard doses of thiopurines. Similarly, individuals with high TPMT activity taking 6-MP or 6-TG may be at higher risk for side effects when treated simultaneously with drugs that inhibit TPMT.

Woodson et al. (1983) studied benzoic acid and a series of its derivatives for their potential to inhibit TPMT. Using the purified human kidney enzyme they found that acetylsalicylic acid and its active metabolite salicylic acid inhibited TPMT. No in vivo studies to confirm the clinical significance of this interaction have been performed. Aminosalicylates, such as sulphasalazine and olsalazine, used in the treatment of ulcerative colitis and Crohn's disease contain benzoic acid in their chemical structures. In several clinical trials, increased 6-TG nucleotide levels have been found in patients with inflammatory bowel disease treated with thiopurines and aminosalicylates compared to thiopurine monotherapy (Lowry et al., 2001; Dewit et al., 2002). In vitro studies using a recombinant human enzyme confirmed that olsalazine, 5-aminosalicylic acid and sulphasalazine are noncompetitive inhibitors of TPMT (Lewis et al., 1997; Szumlanski and Weinshilboum, 1995).

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 trimetoprim-sulphamethoxazole, often used simultaneously with thiopurines in leukaemic patients, had no

effect on TPMT activity in vitro using lysates of red blood cells (RBC) (Jacqz-Aigrain et al., 1994).

Non-steroidal anti-inflammatory drugs (NSAIDs) exhibit pharmacological effects similar to 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 similarly to aminosalicylates and benzoic acid derivatives. The potential for diclofenac, lornoxicam, piroxicam, meloxicam, ibuprofen, ketoprofen, flurbiprofen, naproxen, celecoxib, acetylsalicylic acid, mefenamic acid, tolfenamic acid, metamizole, 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 controls, respectively. The concentration required to inhibit TPMT activity by 50% (IC50), inhibition type and inhibition constant Ki were determined for each compound.

## 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, diclofenac by Pfizer Inc. (MI, USA), and acetylsalicylic acid, metamizole sodium, piroxicam, paracetamol, ibuprofen, 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.** Briefly, erythrocyte TPMT activity was determined in vitro using 6-mercaptopurine (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 $NaH_2PO_4$ 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 DTT (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 haemolysate. Samples were extracted with acetonitrile and 40 μL of extracted sample was injected into the HPLC 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. 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 incubation at 37°C.

**TPMT inhibition studies.** TPMT inhbition studies were carried out using human erythrocytes as a source of enzyme. Haemolysates from 4 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 haemolysates was 118 ng/mL of 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 of 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,

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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.** IC50s were calculated using the WinNonlin inhibitory Imax ordinary or sigmoid library model (WinNonlin 5.0.1, Pharsight Corporation, CA, USA). The goodness 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 $V=Imax*[1-I/(I+IC50)]$, where I is a concentration of inhibitor. For the sigmoid Imax model IC50 was calculated from the equation: $V=Imax*(1-[I^{\gamma}/(I^{\gamma}+IC50^{\gamma})])$, where $\gamma$ 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, CA, USA). 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 $V=[(Vmax*C)/(Km+C)] * Ki/(Ki+I)$, where C is a concentration of substrate and I is a concentration of inhibitor (WinNonlin 5.0.1, Pharsight Corporation, CA, USA).

## RESULTS AND DISCUSSION

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**Determination of IC50 value.** For naproxen, tolfenamic 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 and tolfenamic acid. The Ki values for naproxen (Ki=52 μM), mefenamic (Ki=39 μM) and tolfenamic acid (Ki=50 μM) were very close to the IC50 values obtained in initial experiments which confirms noncompetitive inhibition ($V_{max}$ decreased, but no effect on $K_m$) (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, tolfenamic and mefenamic acid were found to inhibit TPMT with Ki values lower than or comparable with 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 have been determined in human serum. All potential inhibitors were noncompetitive inhibitors of TPMT indicating that TPMT inhibition occurs via binding to the site different from substrate binding

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site. 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 oxidated 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 co-administered 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 effect as adverse events instead of a drug interaction. This may explain the lack of case reports of drug interactions between thiopurines and NSAIDs. A major finding in the current study was that naproxen highly inhibited TPMT activity in vitro. Naproxen has been one of the most commonly used active comparator in clinical trials with selective COX2 inhibitors and appears to be the preferred choice among anti-inflammatory drugs, especially in patients with a high risk of thrombotic events (Antman, et al., 2007). Whether this applies to patients on thiopurine therapy has to be confirmed in further clinical trials.

We used olsalazine as a positive control in our inhibition experiments. In contrast to the previous in vitro study (Ki=12 μM) (Lewis et al., 1997), in our hands olsalazine was a

relatively weak inhibitor of TPMT (Ki=208 μM) applying our experimental conditions. This finding is in correlation with several clinical studies where aminosalicylates therapy was neither a predictor for side effects of thiopurine therapy, low TPMT activity nor 6-MMP levels (Dewit et al., 2002; Gisbert et al., 2006; Hande et al., 2006).

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 have been reported for sulphasalazine 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 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 and mefenamic acid inhibited human TPMT in vitro and clinically significant drug interactions may occur when thiopurines are used simultaneously with various NSAIDs. Further in vivo drug interaction studies are needed to confirm the clinical relevance of the present finding.

## References

Anglicheau D, Sanquer S, Loriot MA, Beaune P and Thervet E (2002) Thiopurine methyltransferase activity: new conditions for reversed-phase high-performance liquid chromatographic assay without extraction and genotypic–phenotypic correlation. *J Chromatogr B* **773:**119-127.

Antman EM, Bennett JS, Daugherty A, Furberg C, Roberts H, Taubert KA (2007) Use of nonsteroidal antiinflammatory drugs: an update for clinicians: a scientific statement from the American Heart Association. *Circulation* **115**:1634-42.

Dewit O, Vanheuverzwyn R, Desager JP and Horsmans Y (2002) Interaction between azathioprine and aminosalicylates: an in vivo study in patients with Crohn's disease. *Aliment Pharmacol Ther* **16:**79-85.

Gisbert JP, Nino P, Rodrigo L, Cara C and Guijarro LG (2006) Thiopurine methyltransferase (TPMT) activity and adverse effects of azathioprine in inflammatory bowel disease: long-term follow-up study of 394 patients. *Am J Gastroenterol* **101:**2769-2776.

Hande S, Wilson-Rich N, Bousvaros A, Zholudev A, Maurer R, Banks P, Makrauer F, Reddy S, Burakoff R and Friedman S (2006) 5-Aminosalicylate therapy is associated with higher 6-thioguanine levels in adults and children with inflammatory bowel disease in remission on 6-mercaptopurine or azathioprine. *Inflamm Bowel Dis* **12:**251-257.

Jacqz-Aigrain E, Bessa E, Medard Y, Mircheva Y and Vilmer E (1994) Thiopurine methyltransferase activity in a French population: h.p.l.c. assay conditions and effects of drugs and inhibitors. *Br J Clin Pharmacol* **38:**1-8.

Lennard L, Rees CA, Lilleyman JS and Maddocks JL (1983) Childhood leukaemia: a relationship between intracellular 6-mercaptopurine metabolites and neutropenia. *Br J Clin Pharmacol* **16:**359-363.

Lewis LD, Benin A, Szumlanski CL, Otterness DM, Lennard L, Weinshilboum RM and Nierenberg DW (1997) Olsalazine and 6-mercaptopurine-related bone marrow suppression: a possible drug-drug interaction. *Clin Pharmacol Ther* **62:**464-475.

Lowry PW, Franklin CL, Weaver AL, Szumlanski CL, Mays DC, Loftus EV, Tremaine WJ, Lipsky JJ, Weinshilboum RM and Sandborn WJ (2001) Leucopenia resulting from a drug interaction between azathioprine or 6-mercaptopurine and mesalamine, sulphasalazine or balsalazide. *Gut* **49:**656-664.

Lysaa RA, Giverhaug T, Wold HL and Aarbakke J (1996) Inhibition of human thiopurine methyltransferase by furosemide, bendroflumethiazide and trichlormethiazide. *Eur J Clin Pharmacol* **49:**393-396.

Oselin K, Anier K, Tamm R, Kallassalu K and Mäeorg U (2006) Determination of thiopurine *S*-methyltransferase (TPMT) activity by comparing various normalization factors: Reference values for Estonian population using HPLC-UV assay. *J Chromatogr B* **13:**77-83.

Shipkova M, Niedmann PD, Armstrong VW, Oellerich M and Wieland E (2004) Determination of thiopurine methyltransferase activity in isolated human erythrocytes does not reflect putative in vivo enzyme inhibition by sulphasalazine. *Clin Chem* **50:**438-441.

Szumlanski CL and Weinshilboum RM (1995) Sulphasalazine inhibition of thiopurine methyltransferase: possible mechanism for interaction with 6-mercaptopurine and azathioprine. *Br J Clin Pharmacol* **39:**456-459.

Woodson LC, Ames MM, Selassie CD, Hansch C and Weinshilboum RM (1983) Thiopurine methyltransferase. aromatic thiol substrates and inhibition by benzoic acid derivatives. *Mol Pharmacol* **24:**471-478.

Xin HW, Fischer C, Schwab M and Klotz U (2005) Thiopurine S-methyltransferase as a target for drug interactions. *Eur J Clin Pharmacol* **61:**395-398.

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# FIGURE LEGENDS

**Figure 1**

Effect of naproxen (A), mefenamic (B) and tolfenamic acid (C) on human TPMT activity in vitro at substrate 6-mercaptopurine concentration of 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM and 1.2 mM. Each of the inhibitor was tested at concentration 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 and tolfenamic acid, respectively, using the noncompetitive inhibition type and simultaneous nonlinear regression (WinNonlin 5.0.1, Pharsight Corporation, CA, USA). Experiments were performed as duplicates.

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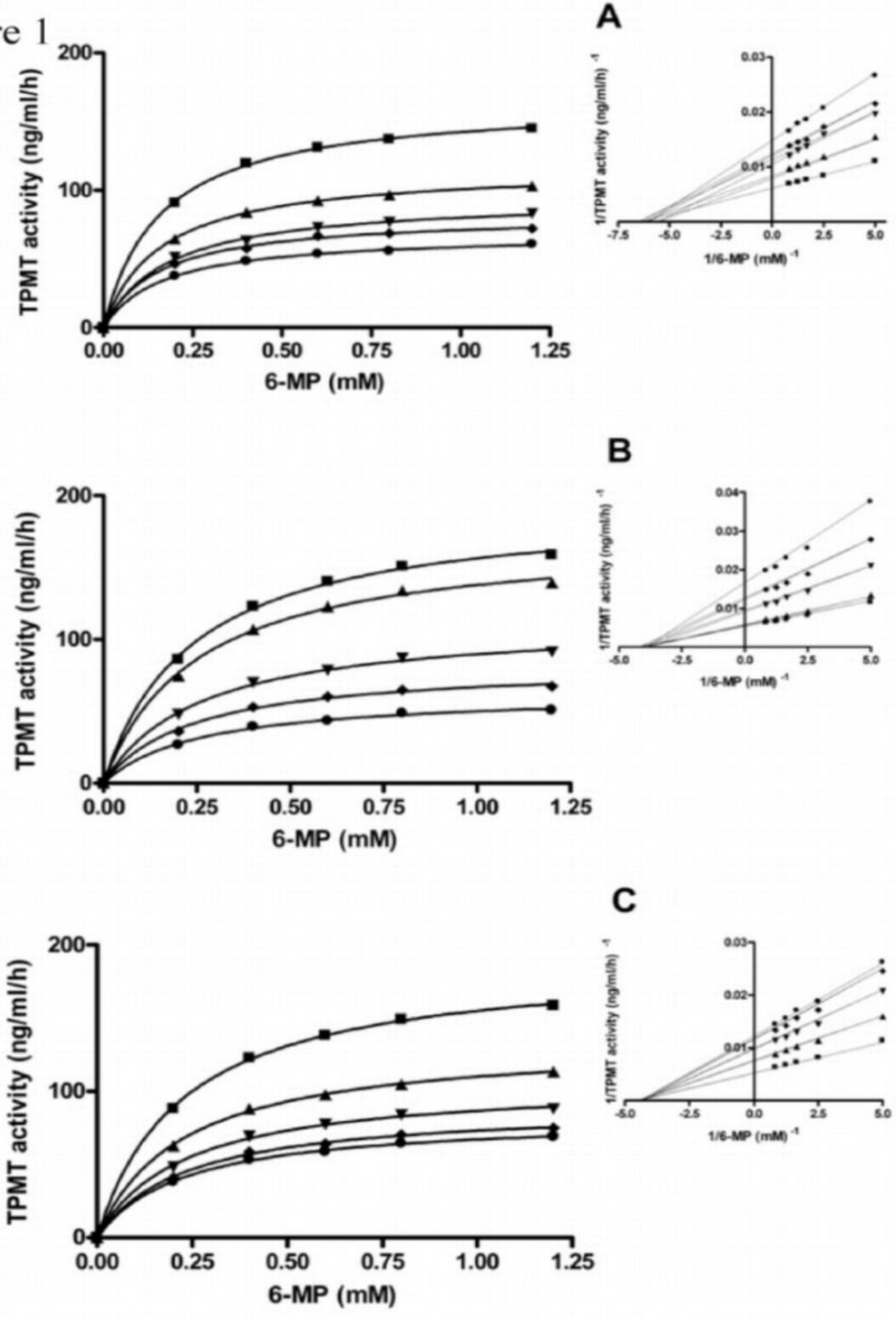
**Table 1.** The IC50 and Ki values for the in vitro inhibition of human TPMT by compounds tested.

| **Compound** | **IC50 value (μM)** | **Ki value (μM)** |
|---|---|---|
| Mefenamic acid | 39 | 39 |
| Naproxen | 79 | 52 |
| Tolfenamic acid | 63 | 50 |
| Olsalazine | 1474 | 208 |
| Ketoprofen | 1013 | 172 |
| Ibuprofen | 1968 | 1043 |
| Lornoxicam | 2135 | 1410 |
| Flurbiprofen | 1649 | 1524 |
| Diclofenac | 1582 | 722 |
| Celecoxib | 2416 | 2413 |
| Piroxicam | 2589 | 2589 |
| Paracetamole | 5168 | 5162 |
| Nabumetone | 4341 | 4300 |
| Meloxicam | 4292 | 4238 |

Figure 1


A
TPMT activity (ng/ml/h)
200
100
0
0.00
0.25
0.50
0.75
1.00
1.25
6-MP (mM)
1/TPMT activity (ng/ml/h) -1
0.03
0.02
0.01
-7.5
-5.0
-2.5
0.0
2.5
5.0
1/6-MP (mM) -1
B
TPMT activity (ng/ml/h)
200
100
0
0.00
0.25
0.50
0.75
1.00
1.25
6-MP (mM)
1/TPMT activity (ng/ml/h) -1
0.04
0.03
0.02
0.01
-5.0
-2.5
0.0
2.5
5.0
1/6-MP (mM) -1
C
TPMT activity (ng/ml/h)
200
100
0
0.00
0.25
0.50
0.75
1.00
1.25
6-MP (mM)
1/TPMT activity (ng/ml/h) -1
0.03
0.02
0.01
-5.0
-2.5
0.0
2.5
5.0
1/6-MP (mM) -1