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Review

Thiopurine *S*-methyltransferase – An important intersection of drug-drug interactions in thiopurine treatment

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ABSTRACT

Understanding the molecular mechanisms of medicines is crucial for developing novel drugs, for repurposing existing medicines, and for predicting toxicities. Thiopurine *S*-methyltransferase (TPMT) serves as an exemplary case in personalized medicine, as its activity is influenced by genetic variants, co-factors, substrates, and inhibitors, which lead to diverse outcomes in thiopurine therapy. This comprehensive review explores the role of TPMT in drug-drug interactions by investigating its interactions with co-factors, substrates, and inhibitors. We focus on the principal interactions of TPMT with clinically relevant inhibitors, and add to this information with molecular docking analyses for the substrate and co-factor binding sites of TPMT. Notably, methotrexate and sulfasalazine emerged as the top-ranked compounds with favorable docking scores for the co-factor binding site, while furosemide is presented as the highest ranked inhibitor for the substrate binding site. Furthermore, we highlight the chemical and structural properties governing ligand binding to TPMT. We support the molecular characteristics by using a summary of clinical implications. Examining the molecular interactions between substrates or inhibitors and TPMT not only addresses therapeutic consequences, but also reveals potential novel indications of interacting compounds. These insights are also invaluable for identifying endogenous ligands and enhancing our understanding of TPMT's biological function.

1. Introduction

The thiopurines 6-thioguanine (6-TG), 6-mercaptopurine (6-MP) and azathioprine (AZA) were developed in parallel with the discovery of the molecular structure of DNA in the 1950s by Nobel laureates, Gertrude B. Elion and George H. Hitchings [1]. They pragmatically acknowledged that the molecules, mimicking natural compounds, can deceive cancer (and immune) cells to readily accept these anti-metabolites, inducing cell death. The thiopurines have become the corner stone of chemotherapy of acute lymphoblastic leukemia (ALL), and an important therapeutic pillar in autoimmune disorders and in the prevention of transplant rejection [2,3].

The interest in thiopurine *S*-methyltransferase (TPMT; EC 2.1.1.67) increased when Lynne Lennard, who had extensively studied thiopurines, and Richard Weinshilboum, who had previously characterized human (h)TPMT, combined forces to describe the role of TPMT in thiopurine metabolism [4]. TPMT was found to catalyse *S*-methylation of thiopurines and other aromatic and heteroaromatic sulfhydryl compounds upon the conversion of its co-factor *S*-adenosylmethionine (SAM) to *S*-adenosylhomocysteine (SAH) [5,6]. SAM not only participates in the transfer of the methyl group, but also has an important role in the stabilization of the enzyme [7]. For a long time, the endogenous function of TPMT was unknown. Recently, significant progress has been made in understanding its biological role. We identified involvement of TPMT in the enzymatically catalysed methylation of selenocysteine [8] (Fig. 1). TPMT was also described to methylate a thiopterine metabolite in the metabolic pathway of a molybdenum co-factor [9]. Furthermore, our transcriptome analysis of over a thousand individuals revealed that TPMT may play a role in oxidation reduction reactions [10].

The understanding of TPMT and drug interactions was built on a

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pleiotropy of studies linking the genomic and metabolomic data with clinical outcomes [13–20]. Detailed molecular mechanisms of TPMT–drug interactions are scarce, with the exception of some crystallographic data and clinical observations of co-therapies with thiopurines [21]. Therefore, we reviewed known interactions between TPMT and pharmacological agents, and delineate the structural characteristics of their bindings. To complement the available information and to further examine the nature of TPMT inhibitor and substrate binding ability, we undertook an *in silico* molecular docking analysis for both the substrate and co-factor binding sites of TPMT with most clinically relevant inhibitors: allopurinol/oxypurinol, sulfasalazine, methotrexate, and furosemide. Based on the computational interactions in terms of scoring functions for multiple binding modes, we illustrate the chemical and structural properties that are responsible for the binding of ligands to TPMT. An understanding of the molecular interactions between known substrates or inhibitors and TPMT provides useful information not only to address the clinical consequences of these interactions, but also for identification of further endogenous ligands and for an understanding of the biological function of TPMT.

2. Thiopurine *S*-methyltransferase: gene to phenotype

The gene that encodes TPMT is located on the reverse DNA strand at 6p22.3 [22]. The starting ATG codon is located within exon 2 and acts as a splice junction which provides most of the mature mRNAs transcribed from eight, rather than nine, exons. The GC-rich promoter region of *TPMT* lacks a TATA or a CAAT box; it includes variable number of tandem repeats (VNTR) which can serve as important *cis*-regulatory elements in gene transcription [23].

According to the TPMT nomenclature committee [24], to date, 46 single nucleotide polymorphisms (SNPs) have been reported and characterized for *TPMT* [25]. The majority of the population carry the wild-type allele, *TPMT*1* (allele frequency 95 % in Europeans). Due to their relatively high frequency and their clinical relevance, the most important SNPs are: 719 A>G in exon 9 (allele *TPMT*3 C;* allele frequency 0.5–1 % in Europeans); 238 G>C in exon 4 (allele *TPMT*2;* allele frequency 0.1–0.2 % in Europeans); and haplotype 719 A>G and 460 G>A in exons 9 and 6, respectively (allele *TPMT*3 A;* allele frequency 3–5 % in Europeans) [26–28]. Allele frequencies differ among different ethnic groups [29]. In addition to SNPs, several VNTR genetic polymorphisms have been reported to date [30–33]. The most common SNPs, *TPMT*3 C* and *TPMT*3 A*, are evolutionarily linked to VNTR motifs with an ABnC pattern [33]. These motifs may contribute to the expression level of the gene, since the binding sites for Sp1, KLF4, and other zinc-finger transcription factors can be predicted from VNTR sequences in the promoter region of *TPMT* [34].

Individuals differ with regard to TPMT activity. The population distribution of TPMT activity in people of European descent is trimodal: around 89 % of individuals have normal enzyme activity of TPMT; 11 % have intermediate; and ~0.3 % have almost undetectable TPMT activity [35]. The activity of TPMT depends on genetic variants in *TPMT* [4,19,36], as well as other factors involving concentration of methyl donor SAM and variants in genes of methionine and the folate cycle [7,37–39].

Human TPMT (hTPMT) is a monomeric enzyme that comprises 245 amino-acid residues, with a molecular weight of 28.18 kDa. This single-domain protein consists of eight α helices and nine β sheets that are symmetrically distributed around the central β1 sheet [11]. The outer structure of TPMT is rigid and resembles the structure of other class I methyltransferases [11,40], while the area around the active site of TPMT is more flexible and specific for methylation of thiopurines [11,21]. hTPMT contains two binding sites that are positioned near each other, one of which binds the co-factor SAM, and the other binds the substrate [11,21]. The activity of TPMT is determined by the interaction between the methyl donor SAM and the substrate, followed by the transfer of a methyl group from SAM to the substrate [11,21,40].

Post-translational stabilization enabled by the co-factor and methyl

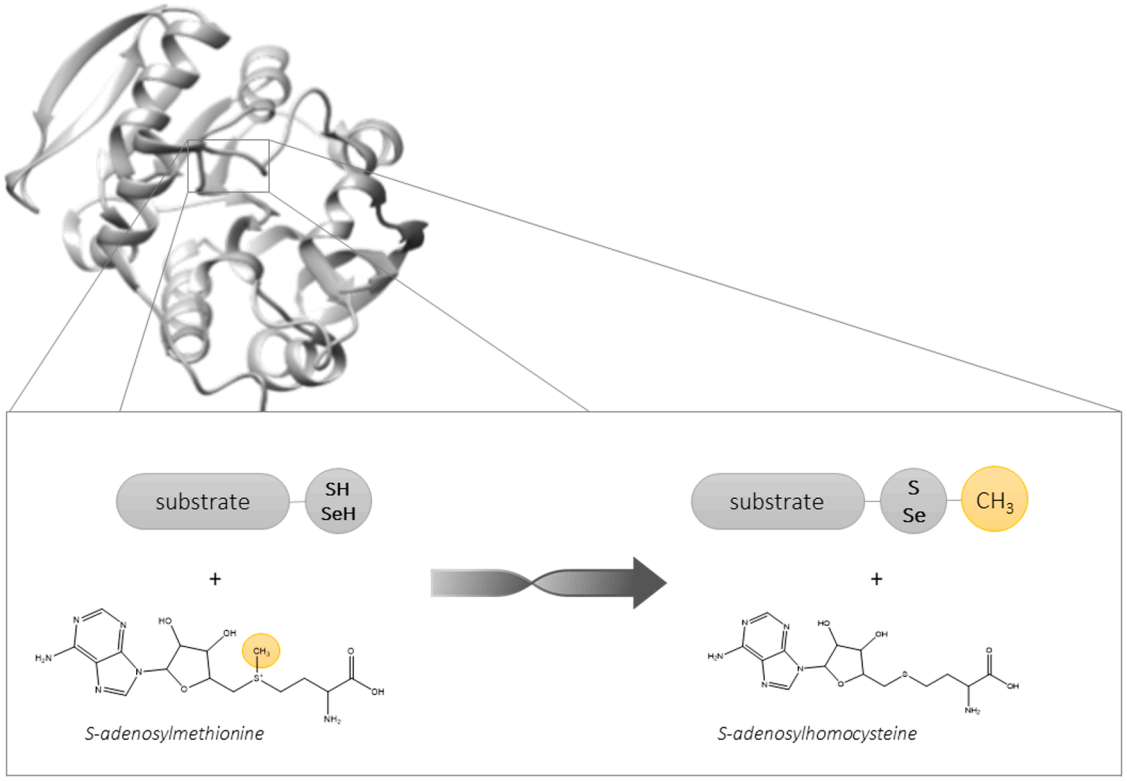

Fig. 1. A reaction catalyzed by thiopurine *S*-methyltransferase (TPMT). Upon TPMT binding, *S*-adenosylmethioinine (SAM) transfers the methyl group to the substrate. After methylation, the methylated substrate and *S*-adenosylhomocysteine (SAH) leave the active site of TPMT. The TPMT structure was obtained from crystallographic data in PDB ID 2BZG [11,12].

donor SAM is another important factor affecting TPMT activity [7,41]. A study on TPMT from *Pseudomonas syringae* showed that sinefungin, an analogue of SAM, induces conformational changes in the active site of TPMT and stabilizes the peripheral part of the TPMT backbone [42]. SAM has also been shown to inhibit the degradation of variant TPMT by blocking the interaction between the chaperone hsp90 and TPMT [43].

In a large population study, we demonstrated that higher levels of SAM correspond to higher TPMT activity in erythrocyte lysates [41]. By selectively disrupting enzymes involved in methionine cycle, we have shown that sufficient SAM levels are important in preserving TPMT stability, and consequently enzymatic activity [7]. Furthermore, when applying the cytostatic drug and TPMT substrate 6-MP to cultured cells, we observed that the addition of SAM reduced cytotoxicity of 6-MP, and prolonged the viability of MOLT lymphoblasts, which are an *in vitro* model for ALL [39]. We also translated this finding to the clinical level, where we identified genetic polymorphisms in genes involved in the methylation pathway, *MTHFR* and *TYMS*, as potential biomarkers for increased thiopurine toxicity in patients with ALL [37,38].

2.1. Structural features of thiopurine S-methyltransferase interactions with a co-factor and substrate

The crystallographic structures of murine (m)TPMT and hTPMT were resolved by Peng and Wu, respectively [11,21]. The structures are available in the protein data bank (PDB) under IDs 3BGD [21,44] and 2BZG [11,12], and give important insights into TPMT structural characteristics and the interactions between TPMT, its co-factor, and 6-MP. Based on the crystallographic data, we know that TPMT contains two binding pockets in its active site – one for the co-factor SAM and the other for the substrate – and a flexible loop, lying near the active site (Fig. 2). The pockets are interconnected on the site where the methylation reaction takes place. Upon the transfer of the methyl group from SAM to the substrate, *S*-adenosylhomocysteine (SAH) and methylated substrate are formed (Fig. 1). Crystallographic data describing TPMT structure and the illustrative features of the active site provided an excellent basis for an *in silico* molecular docking analysis of selected inhibitors that we present in the last part of this review.

The binding site for SAM is wide and easily accessible (Fig. 2A) as in other SAM-dependent methyltransferases. The entrance to the channel that binds SAM consists of acidic amino acids [11], which can facilitate the access of the positively charged co-factor. The interactions between SAM and TPMT are strong, and they involve several hydrogen bonds, with the adenosine part captured in a firm sandwich structure between Ile135 and Ile90 [11,21], which contributes to the high affinity of this binding (K_m = 2.7 μM) [45].

However, the binding site of 6-MP is specific to TPMT (Fig. 2B) and contains two channels through which the substrate can either enter or exit TPMT. In the binding site, 6-MP does not form direct hydrogen bonds with TPMT; instead, it binds to TPMT with hydrophobic and van der Waals interactions that are formed by phenylalanine (Phe40), prolines (Pro195, Pro196), and two arginine residues (Arg152, Arg226) in the active site [11,21]. The affinity towards 6-MP is thus significantly lower compared to SAM (K_m = 300 μM) [45,46].

A flexible loop and an α helix are positioned in close proximity to the active site of TPMT. For mTPMT, the α helix lies between the Ser34 and His47 residues and the Glu220 and Trp225 residues. In hTPMT, these correspond to the Ala39 and His52 residues and the Glu225 and Trp230 residues (Fig. 2C). The α helix can induce conformational changes to the active site, which provides closer contact between the substrate and co-factor [11,21]. On account of this flexibility in the active site, TPMT might be a target for both smaller and bulkier substrates compared to 6-MP and its metabolites [11,21].

Molecular docking of 6-MP with hTPMT and results from the mTPMT co-crystalized with 6-MP provide insights into the catalytic mechanism of methylation by TPMT [11,21]. Docking results indicate that in hTPMT, the sulfur atoms of 6-MP and SAM are arranged linearly, with

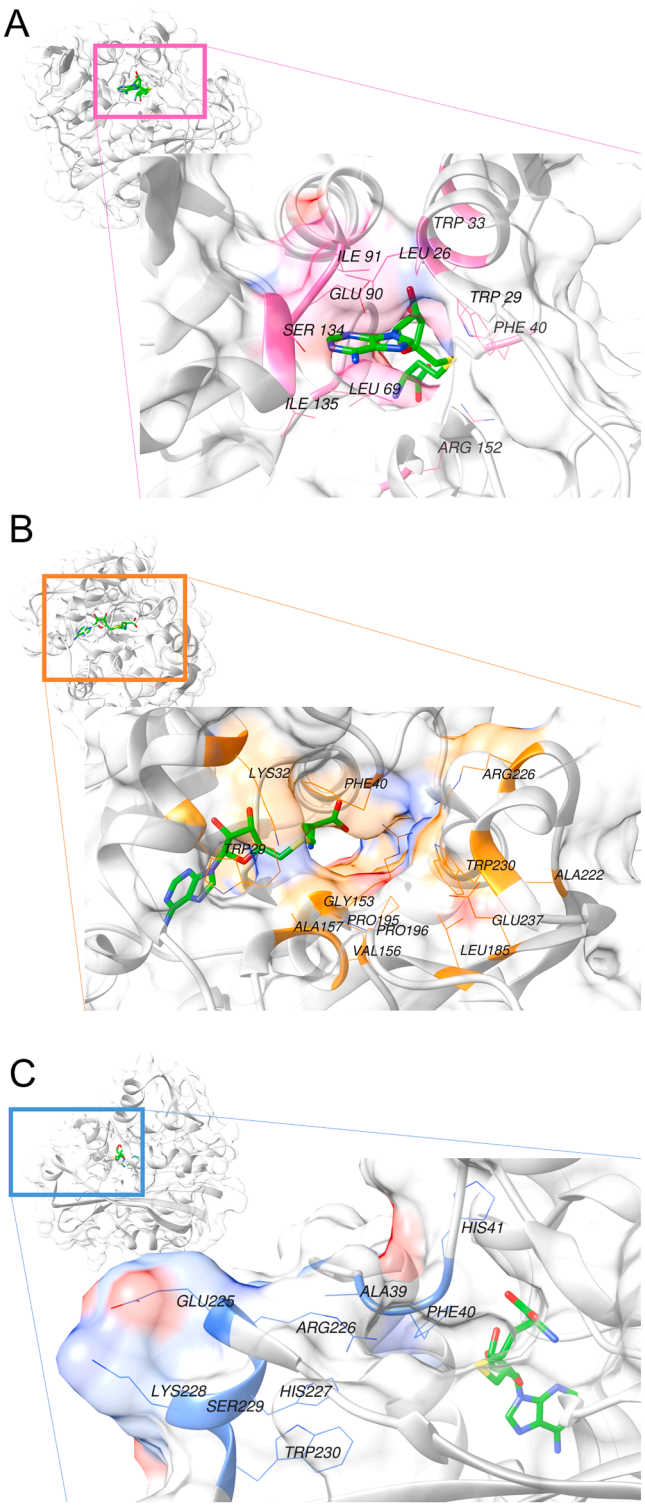

Fig. 2. Basic features of the binding sites and a flexible loop of recombinant human TPMT, highlighting amino acid residues involved in co-factor and substrate binding for the methylation reaction (PDB ID: 2BZG [11,12]). (A) The entrance to the binding site for SAM/SAH (binding site, magenta; SAH, green). (B) The perspective from the 6-MP binding site (orange). (C) The loop and α helix, which show flexibility upon the substrate binding (blue). 6-MP, 6-mercaptopurine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; and TPMT, thiopurine *S*-methyltransferase.

the sulfur atom on 6-MP oriented to attack the methyl group on SAM, supporting a classic S_N2 mechanism of the methylation reaction [11]. This finding is consistent with observations on mTPMT, where the sulfur atoms of co-crystalized SAH and 6-MP are 1.8 Å apart, further confirming the direct S_N2 mechanism [21], characteristic for most SAM-dependent methyltransferases [47]. No charged or polar amino acid residues are located near the target sulfur atom on 6-MP, corroborating the direct transfer of the methyl group from SAM to 6-MP. In the hTPMT structure, Lys32 and Arg226 are positioned near the superimposed 6-MP, facilitating interactions with the nitrogen atoms on 6-MP [11]. These interactions may enable deprotonation of the nitrogen (either N7 or N9), producing a negatively charged S^- ion, which is anticipated for the subsequent nucleophilic substitution in this methylation reaction [11,21]. In mTPMT, Arg221 and Arg147 were identified as the possible residues responsible for the deprotonation of 6-MP [21].

3. Substrates of thiopurine *S*-methyltransferase

Exogenous substrates – cytostatic and immunosuppressive drugs, thiopurines – gave identity to TPMT [40,48] and raised interest in finding other substrates for TPMT that focus the research on structurally similar compounds. This led to substrate identifications among several synthetic compounds, as well as compounds found in biological systems (Table 1) [6,8,45,46,48–50]. In the late 1980s, the enzyme kinetics of methylation of aliphatic and aromatic thiol compounds were evaluated using TPMT, followed by the enzyme kinetics measurements for the methylation of purines, pyrimidines, and their analogues in the 1990s. With the development of more sensitive and selective analytical methods in the last few years, endogenous selenium compounds were identified to be methylated by TPMT.

Compared to 6-MP, fast methylation kinetics have been reported for thiophenol derivatives, with apparent K_m values ranging from 0.29 to 7.8 μM (v_{max} was not calculated) [6,51]. The experiments were performed on purified human kidney TPMT. The same source of TPMT was used when purine analogues were analyzed, which allowed comparison between different bioisosteric replacements of oxygen on site 6 of the purine ring [46]. Among purine analogues, 6-selenopurine exhibited the lowest K_m value (29.1 μM) and one of the highest v_{max} values (1950 U/mg TPMT). 2-Thiouracyl has also been reported to be a substrate for TPMT, with enzyme kinetics comparable to those of 6-MP [45,48]. Recently, selenocysteine and a thiopterin metabolite were reported as substrates for TPMT [8,9]. Sulfhydryl aliphatic compounds, with the exception of diethyldithiocarbamate (apparent K_m 7.9 μM and v_{max} 35 U/mg TPMT), showed little or no interaction with TPMT [6,8], and so TPMT does not appear to influence the metabolism of endogenous purines [52].

Table 1 summarizes the kinetic parameters (K_m, v_{max}) for these substrates. For specific substrates, different kinetic parameters have been reported, as the kinetic analyses were performed with such a diverse set of methods and many different sources of TPMT. To allow at least slight comparison of these parameters, the table also includes the sources of TPMT used in each study. We review thiopurines and selenium compounds as substrates for TPMT in detail in the following sections of this review, as thiopurines are clinically and significantly interconnected with TPMT [19,20], and the emerging studies on selenium metabolism demonstrate TPMT involvement in methylation of selenium compounds [8,50].

3.1. Thiopurines

Despite advances in therapeutic approaches, the use of the thiopurines (6-MP, 6-TG, and AZA) is still of crucial importance for the maintenance of immune system equilibrium to combat ALL, inflammatory bowel disease (IBD), and other autoimmune disorders [20,56,57]. After their administration, thiopurines undergo a complex metabolism, which consists of both activation and deactivation (Fig. 3) [58]. In the intestine and liver, AZA is partly enzymatically cleaved by glutathione that forms methylnitrothioimidazole and 6-MP. The 6-MP then enters the purine salvage pathway that is directed towards cytotoxic metabolites. Transformation of 6-MP by hypoxanthine phosphoribosyl transferase, inosine monophosphate dehydrogenase, guanosine monophosphate synthase, and kinases yield active 6-thioguanine nucleotides (6-TGNs) [59,60]. The deactivation route of 6-MP constitutes two major enzymes, TPMT and xanthine oxidase, which yield 6-methylmercaptopurine (6-MMP) [48] and thiouric acid [61], respectively.

Due to their structural similarities, thiopurines participate in cellular processes that antagonize those of the endogenous purine nucleotides (Fig. 3). Incorporation of 6-TGNs into nucleic acids results in the arrest of polymerase-driven DNA extension and DNA breakage [58,62–64]. The 6-TGNs also interfere with DNA duplication through inhibition of DNA polymerase and DNA ligase, as well as RNA transcription, by modulation of RNase H activity [65]. By mimicking GTP, the 6-TGNs

Table 1
Known substrates with kinetics parameters for thiopurine *S*-methyltransferase.

	K_m [μM]	V_{max}	Source of TPMT	Ref
Thiopurines and their analogues				
6-MP	10.6	48*	4	[53]
	383	897**	1	[46]
	200		3	[48]
	680			[21]
	265.4	126***	5	[8]
	120	68****	6	[54]
6-MP-riboside-monoP	1270	1630**	1	[46]
	25.7	31*	4	[53]
6-MP-riboside	1170	641**	1	[46]
	55.1	89*	4	[53]
6-MP-riboside-triP	890	100**	1	[46]
6-thiodeoxyinosine	12.7	22*	4	[53]
8-hydroxi 6-MP	96.1	1630**	1	[46]
7-methyl 6-MP	231	2340**	1	[46]
9-(n-buthyl)−6-MP	292	1770**	1	[46]
9-ethyl−6-MP	372	1460**	1	[46]
6-hydroxi−8-MP	138	324**	1	[46]
6-TG	18.1	55*	4	[53]
	557	1080**	1	[46]
	156	86****	6	[54]
6-TG-riboside	26.1	32*	4	[53]
	761	388**	1	[46]
6-TG-riboside-monoP	27.1	59*	4	[53]
	1040	75**	1	[46]
6-thiodeoxyguanosine	131.4	120*	4	[53]
Selenium compounds				
6-selenopurine	29.1	1950**	1	[46]
6-selenopurine riboside	51.8	1990**	1	[46]
6-selenoguanine riboside	139	1250**	1	[46]
Selenocysteine	355	52***	5	[8]
Other compounds				
Thiophenol derivatives	0.29 – 7.8¥	n.a.	1	[6,51]
2-thiouracyl	1700	n.a.	3	[48]
	2000	217**	1	[55]
Thiopterin	1.86	n.a.	5	[9]
Dithiocarbamate	7.9¥	35**	2	[49]
2-mercaptoethanol	$168 \cdot 10^3$	128**	1	[45]

1 purified human kidney TPMT
2 human liver microsomal and cytosolic extracts
3 mouse kidney homogenates
4 human recombinant TPMT expressed in yeast
5 human recombinant TPMT expressed in E. coli
6 Erythrocyte lysates
¥ apparent K_m
n.a. not available
6(M)TG, 6-(methyl)thioguanine; 6(M)MP, 6-(methyl)mercaptopurine; Hb, haemoglobin; SAM, *S*-adenosylmethionine; TPMT, thiopurine *S*-methyltransferase.
* nmol/min/mg of TPMT
** U/mg TPMT
*** pmol SAM/min/mg recombinant TPMT
**** 6MTG (or 6-MMP)/gHb h

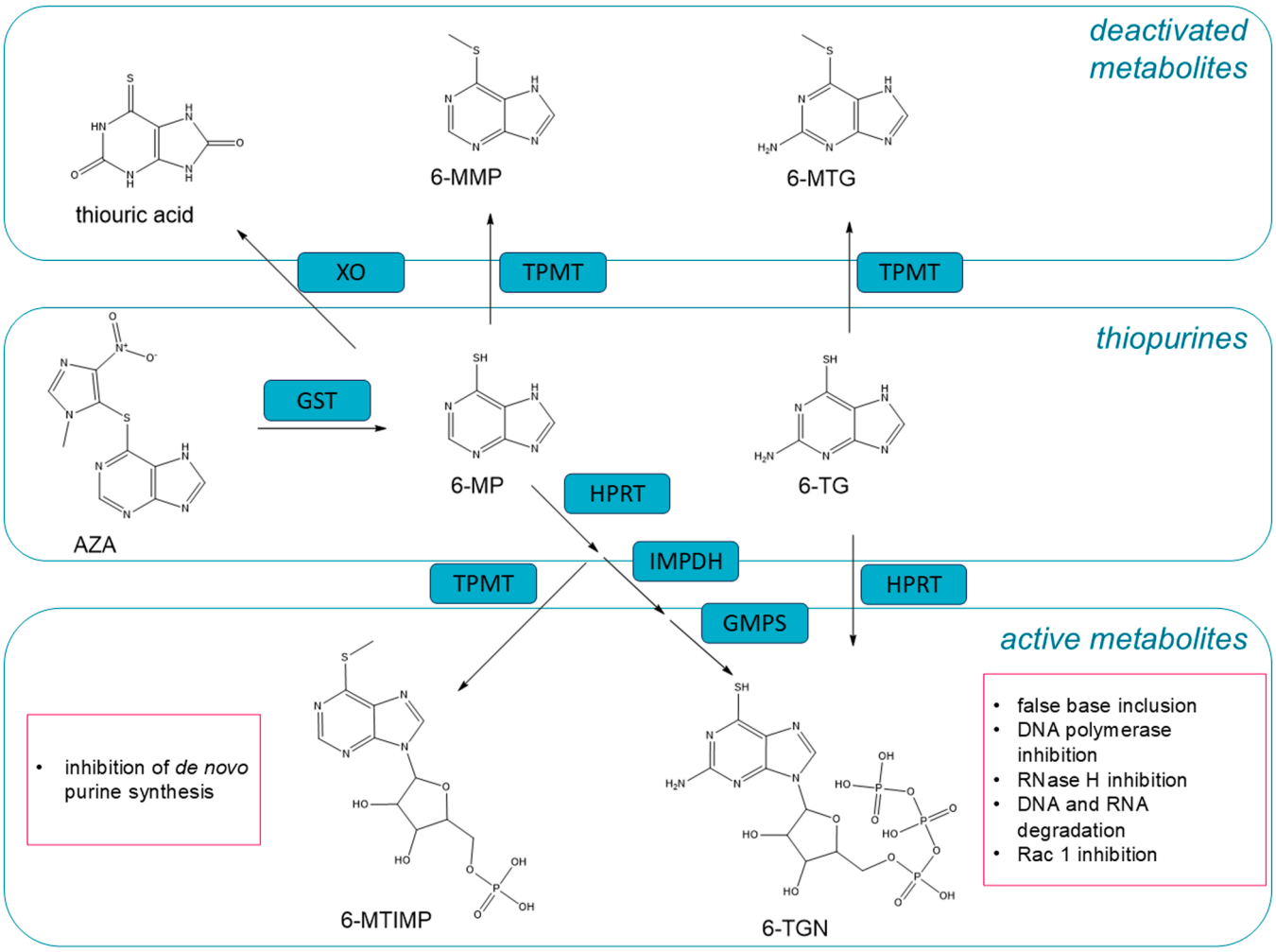

Fig. 3. Schematic representation of thiopurine metabolism and mode of action. 6-MP, 6-mercaptopurine; 6-MMP, 6-methylmercaptopurine (analogues); 6-MTG, 6-methylthioguanine; 6-MTIMP, 6-methylthioinosine monophosphate; 6-TG, 6-thioguanine; 6-TGN, 6-thiguanosine nucleotides; AZA, azathioprine; GMPS, guanosine monophosphate synthase; GST, glutathione transferase; HPRT, hypoxanthine phosphoribosyl transferase; IMPDH, inosine monophosphate dehydrogenase; TPMT, thiopurine *S*-methyltransferase; XO, xanthine oxidase.

block CD28-dependent Rac1 activation and direct T lymphocytes towards apoptosis [66]. Along with the 6-TGNs, methylated products of 6-MP trigger cell arrest through obstruction of *de-novo* purine synthesis (Fig. 3) [67]. A by-product of AZA degradation, methylnitrothioimidazole, has also been associated with further immunosuppressive effects of currently unknown mechanism [68,69].

Since TPMT is an important deactivating enzyme for thiopurines, genetic polymorphisms that affect TPMT activity significantly interfere with thiopurine efficacy and toxicity in patients [70]. In a number of clinical studies, the presence of genetic polymorphisms and lower TPMT activity have been acknowledged as factors for increased risk of myelosuppression when these patients received the normal dose of thiopurines [4,14,17,71–73]. Furthermore, TPMT status strongly correlates with dose reduction and cessation of thiopurine treatment [72–76]. Such extensive studies have provided a lot of good evidence for TPMT genotype–phenotype correlations. The current guidelines of Clinical Pharmacogenetics Implementation Consortium (CPIC), Canadian Pharmacogenomics Network for Drug Safety or of Dutch Pharmacogenetics Working Group (DPWG), thus strongly recommend the use of genetic information on *TPMT* to adjust the prescribing of thiopurines [77]. The starting dose for patients heterozygous for *TPMT* or patients with intermediate TPMT activity should be 30–80 % of a normal thiopurine dose. For poor metabolizers, a 10-fold reduction of the normal starting dose or the replacement of the therapeutic agent is required [70].

3.2. Selenium compounds

As analogues of thiopurine bases, selenopurines exhibit strong antiproliferative activities. While thiopurines have been included in treatment protocols for leukemia and autoimmune diseases for decades, the toxicological profile of 6-selenopurine was not satisfactory for the compound to be included in further clinical studies [78–80]. Systematic research into the methylation of purine analogues has revealed that 6-selenopurine exhibit one order of magnitude higher affinity and a higher TPMT methylation rate (K_m 29.1 μM and v_{max} 1950 U/mg TPMT) compared to 6-MP (K_m 383 μM and v_{max} 897 U/mg TPMT) [46].

In agreement with this finding and in the pursuit of understanding endogenous role of TPMT, we recently provided evidence that selenocysteine is a substrate for hTPMT [8]. Selenocysteine (Sec) is an essential part of selenoproteins, through which selenium plays an indispensable role in oxidative reactions, the synthesis of thyroid hormones, fetal development, and many more physiological processes [81,82]. The Selenol group (SeH) on Sec is a highly reactive functionality due to its nucleophilic nature and certain selenium-accumulating organisms developed methylation mechanisms for its deactivation [83-86]. We discovered that selenocysteine binds to hTPMT with a positioning that promotes the transfer of the methyl group from SAM. We demonstrated formation of *Se*–methylselenocysteine (MeSec) in this enzymatic reaction, which progresses within the same range of kinetics as seen for 6-MP

[8]. This finding suggests that the transformation is also viable for biological systems. An additional indication of endogenous TPMT involvement in selenium metabolism comes from a study in which bacteria with highly active TPMT orthologues produced significantly higher levels of methylated selenium compounds (i.e., dimethylselenide, dimethyldiselenide) when supplemented with selenium compounds, such as selenite, MeSec and selenate, in fresh water, as well as in cell cultures [87–89]. Selenium methylation by TPMT was further characterized in two very recent studies, suggesting TPMT as selenium methyltransferase preferentially for non-methylated and monomethylated negatively charged forms of selenium [50,90].

Beside selenium compounds, the TPMT orthologue from *Pseudomonas syringae* and TPMT-like enzymes (e.g., tehB) have been recognized as factors for bacterial resistance to tellurite compounds [91,92], indicating a possible implication of TPMT in tellurite detoxification.

3.3. Structural characteristics of thiopurine S-methyltransferase substrates

The general structure for TPMT substrates includes a thiol or selenol group (Fig. 4, purple) attached to a (hetero)aromatic ring (Fig. 4, blue). Due to the stabilization of the thiolate anion and the acidity of such compounds (e.g., thiophenol pKa, 6), they can readily react with alkylating agents in a neutral, or even in a slightly acidic, environment [6, 53].

In addition to enhancing the reactivity of the thiol group, the pyrimidine part of the ring in thiopurines has an important role in π–π bond formation with Phe35 in the active site of mTPMT (Phe40 in hTPMT), as indicated by the position of 6-MP when co-crystallized with mTPMT. The hydrophobic surface of TPMT thus 'captures' the ring, while two positively charged key arginine residues in the substrate binding site form interactions with slightly electronegative nitrogen atoms of imidazole (i.e., N7, N9), which enables the deprotonation of 6-MP [21]. The substituents on the rings (Fig. 4, orange) might further modulate this reaction in different ways. As can be seen from the data in Table 1, ribose and the accompanying phosphates at position 9 of the purine ring decrease the affinity of TPMT towards 6-thiopurines when compared to their unribosylated form (e.g., Table 1, K_m increase from 383 μM to 1270 μM) [46]. Hydroxy, methyl, t-butyl and ethyl groups in positions 7, 8, or 9 on the purine ring do not markedly interfere with the methylation kinetics of the thiol group on 6-MP. The K_m and V_{max} did not change to any great extent after these modifications (Table 1) [46]. The thiopterin structure, on the other hand, vastly enhances enzyme kinetics [9]. Extensive studies on thiophenol derivatives have generally been inconclusive in terms of the influence of modifications [6,51].

Compared to a thiol group, a selenol group on the selenopurines represents a much stronger nucleophile, which thus defines more rapid enzyme kinetics for TPMT. Indeed, the affinity of 6-selenopurine towards TPMT is higher compared to 6-MP by one order of magnitude (Table 1, K_m decrease to 29 μM from 383 μM, respectively,) [46]. Furthermore, selenol functionality is a sufficient nucleophile in the methylation reaction even without the aromatic ring, as the second dissociation constant pK_2 of the aliphatic selenols are lower than those of aliphatic thiols (pK_2 selenocysteine, 5.2; pK_2 cystine, close to 8.6) [85, 86,93].

4. Inhibitors of thiopurine *S*-methyltransferase

A number of medicines regularly used in clinical practice have been shown to modulate TPMT activity (Fig. 5, Table 2). Interactions of TPMT with drugs, intermediates, and metabolites can consequently affect outcomes of thiopurine treatment. Among pharmacological agents, the most potent inhibitors of TPMT according to the *in vitro* TPMT kinetics characterization are furosemide, olsalazine, and sulfasalazine [94–97], followed by non-steroidal anti-inflammatory drugs (NSAIDs; e.g., naproxene, mefenamic, tolfenamic acid) [98], oxypurinol, a metabolite of allopurinol [99], other 5-aminosalicylates (e.g., balsalazine, 5-aminosalicylic acid – 5-ASA [mesalazine], N-acetyl-5-ASA) [95–97] and thiazide diuretics (e.g., bendroflumethiazide, trichlormethiazide) (Table 2) [94]. Among the products of thiopurine methylation, SAH and 6-MMP, inhibit TPMT activity (Table 2) [35]. Thioxanthine, a metabolite of thiopurines that is increasingly produced when thiopurines are co-administered with allopurinol, also inhibits TPMT [99]. Most relevant clinical examples of TPMT-interacting drugs, involving alopurinol, aminosalicylates, methotrexate, NSAIDs and furosemide, are discussed below.

4.1. Allopurinol and oxypurinol

Co-administration of allopurinol to overcome thiopurine resistance has been used in clinical practice, especially in the treatment of refractory IBD. As an inhibitor of xanthine oxidase (Fig. 5), allopurinol represents a mainstay in the prevention of gout and other conditions characterized by hyperuricaemia, although it was first synthesized as a candidate molecule for chemotherapy [103]. Allopurinol was used for leukemia patients on thiopurine therapy in an attempt to ameliorate thiopurine availability [104–106]. Despite initially unsuccessful efforts to use allopurinol as a companion to thiopurines that arose because of severe toxicity of the co-treatment [107], clinicians have endeavored to demonstrate the potentiation of the thiopurine efficacy in co-therapies in recent decades.

4.1.1. Interactions with thiopurine S-methyltransferase

Allopurinol does not directly inhibit TPMT [98]; however, *in vitro*, it has shown weak mixed competitive inhibition with its metabolite oxypurinol, with an apparent K_i of 1.186 mM (Fig. 3, Table 2) [99,108]. Allopurinol co-administration with 6-MP also induces enhanced production of thioxathine (Fig. 5) [99], which is a known inhibitor of recombinant TPMT, with an apparent K_i of 0.329 mM (Table 2) [54,99]. Higher levels of thioxanthine in blood of thiopurine treated patients correlate with improved 6-MMP/6-TGN ratio as determined in clinical investigations of allopurinol–thiopurine co-therapies [109,110].

4.1.2. In silico molecular binding mode analysis

To complement the information on the structural characteristics of the inhibitor binding, we performed molecular docking analyses for oxypurinol, aminosalicylates, methotrexate, and furosemide for both substrate and co-factor binding sites of TPMT. After the *in silico* construction, optimization and minimization of the compounds, and preparation of the receptor molecule (see details in Supplementary Information), we performed docking on a crystal model of mTPMT (PDB entry: 3BGD [21,44]). Despite a well-established crystal model for hTPMT (PDB ID: 2BZG [11,12]), to date, the crystal structure of mTPMT (PDB ID: 3BGD [21,44]) is the only reported structure with the co-crystalized substrate (6-MP) bound to the substrate binding site. This allows for better comparisons and evaluations in investigations of ligand binding. The attainability of binding was evaluated using scoring functions.

The kinetics data on oxypurinol inhibition do not indicate its precise type of TPMT inhibition. Therefore, in our molecular docking analysis, we examined possible oxypurinol bindings to both the substrate and co-factor binding sites of TPMT. Due to the small size of oxypurinol molecule and the multiple calculated binding modes, a cluster analysis was carried out to determine the observed binding pattern (Supplementary Information (SI), Figure S1). In one of the clusters, where oxypurinol was analogously positioned to the co-crystallized 6-MP (mTPMT; PDB ID: 3BGD [21,44]; Cluster 2; RMSD: 1.38 Å), all of oxypurinol binding modes in the substrate binding site occupied the electron density of the co-crystallized substrate (Fig. 6A, left, Figure S1). Alternative binding modes had a flipped conformation, which indicated that the pyrazole moiety on oxypurinol roughly occupied the space of the pyrimidine moiety on the co-crystallized 6-MP, while the pyrimidine moiety on oxypurinol filled the space of imidazole on 6-MP (Clusters 4,

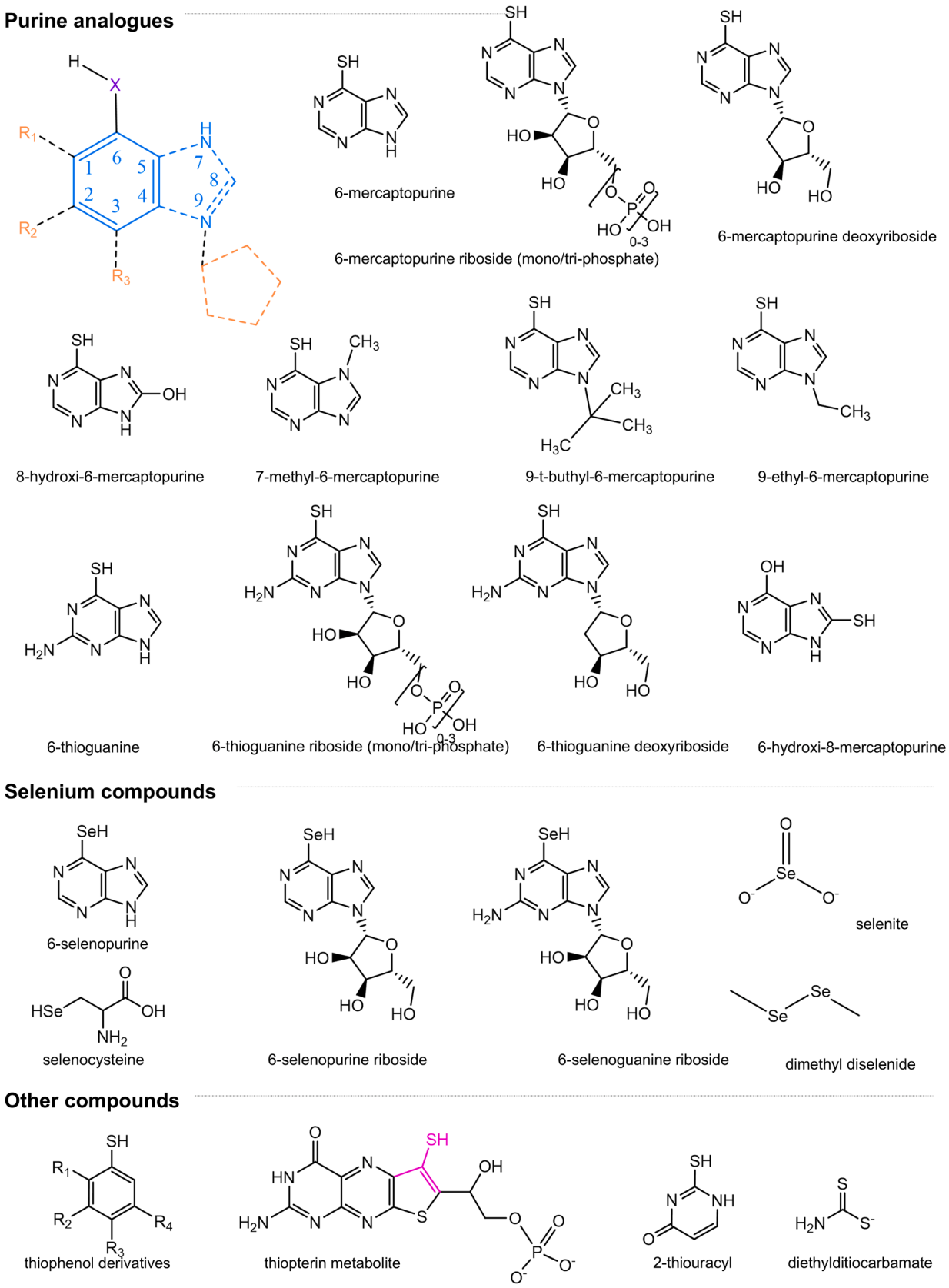

Fig. 4. Schematic representations of thiopurine *S*-methyltransferase substrate features (upper left corner) and structures. Magenta – uncertain part of the structure.

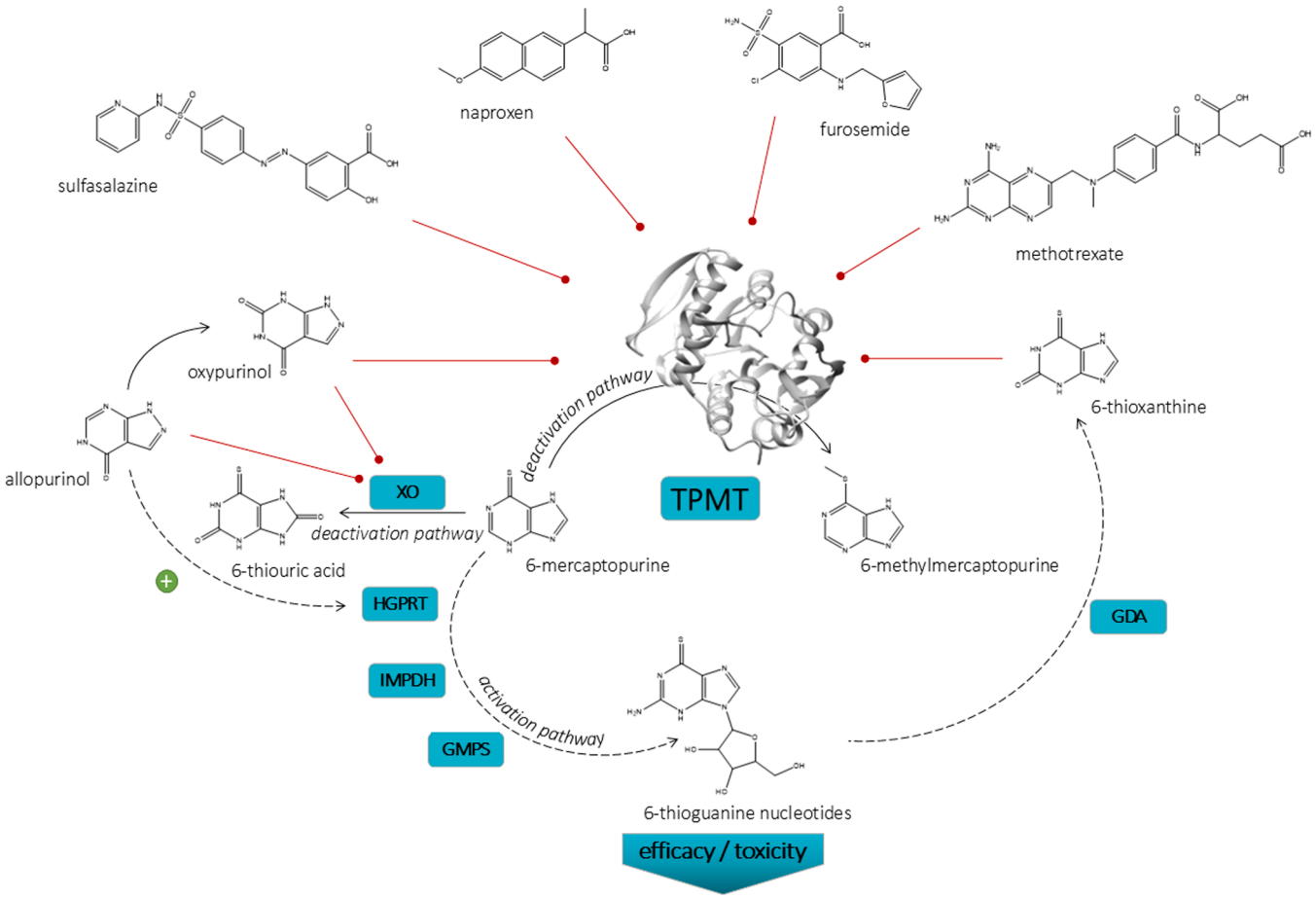

Fig. 5. Schematic representation of main inhibitors of thiopurine *S*-methyltransferase and their mode of action (PDB ID 2BZG [11,12]). Red line: inhibition of targeted enzyme; green: enhanced activity towards targeted enzyme; dashed lines: transformation involves more than one step; GDA, guanine deaminase; GMPS, guanosine monophosphate synthase; HGPRT, hypoxanthine phosphoribosyl transferase; IMPDH, inosine monophosphate dehydrogenase; TPMT, thiopurine *S*-methyltransferase; XO, xanthine oxidase.

5; RMSD: 4.41, 3.77 Å). In the latter binding modes, oxypurinol still preserved its key interactions with Ser34, Phe35, and Arg221 of mTPMT. Clusters 1 and 3 resulted in a 90°-rotated conformation of oxypurinol with respect to the co-crystallized 6-MP, where its pyrimidine-2,4-dione moiety still effectively interacted with Ser34 and Arg221. Considering all of the binding modes, we can conclude that oxypurinol can effectively occupy the substrate binding site of TPMT and occlude access of the substrate to the co-factor. This analysis also suggested ample space for positioning of larger ribosylated metabolites of oxypurinol.

After the docking of oxypurinol to the co-factor binding site of TPMT (mTPMT; PDB ID: 3BGD [21,44]), one dominant pose was observed (Fig. 6A, right). Oxypurinol occupied the space of the adenosine moiety of the co-crystallized SAH, the demethylated co-factor. The pyrimidine-2,4-dione oxypurinol residue interacted with Glu85, Ile86, Ser129 and Leu21 of mTPMT, whereas the pyrazole ring extended towards the ribose of the co-crystallized SAH, to interact with Trp24, Leu64 and Glu85 of mTPMT. The docking score for oxypurinol in the TPMT co-factor binding site (Fred score, −9.165 kcal/mol) was relatively favorable, which indicated both of these binding sites as viable options for the interactions between TPMT and oxypurinol.

4.1.3. Clinical implications

Allopurinol was first included in ALL treatment in co-therapy with thiopurines several decades ago [104]. High doses of allopurinol resulted in severe thiopurine-related toxicities provoking the withdrawal of allopurinol in this co-treatment [107]. The first successful attempt of allopurinol-thiopurine treatment, was a low-dose allopurinol that potentiated the immunosuppressive effect of triple therapy including AZA, cyclosporine, prednisolone in renal transplantation patients [111]. Both, the dose of AZA sufficient to reach response and the number of rejection episodes were significantly reduced by administering allopurinol [111]. Based on these observations, the synergistic activity of thiopurines and allopurinol was further exploited for adult and paediatric patients with IBD [112–116], and corroborated by a recent large retrospective population-based cohort study [117]. Such a combination allows significant reduction of thiopurine dose (to 23 %–30 % standard dose) while (still) achieving therapeutic 6-TGN concentrations [118,119]. Furthermore, it decreases 6-MMP levels, and, consequently, mitigates hepatotoxic events, and improves rates of clinical response (up to 100 %) and long-term remission (from 72 % to 82 %) [110,117,119–123]. Additionally, thiopurine and allopurinol co-therapy in IBD has been recognized as significantly cost-effective [124]. Studies have shown the beneficial effects of low-dose allopurinol administration in co-therapy with 6-MP of ALL patients [109,118,125], as well as in patients with autoimmune hepatitis [57,126–129].

The results of allopurinol and thiopurine co-treatments indicate that emerging protocols, if not already, will include this drug combination for unresponsive patients or for those who suffer from severe thiopurine-induced side effects. Further experimental structural insights and detailed biochemical evaluations are needed to fully define the inhibitory mechanism of oxypurinol against TPMT. However, health-threatening pancytopenia and other complications in gout patients or patients with genetic polymorphisms in *NUDT15* taking allopurinol and

Table 2
Thiopurine *S*-methyltransferase inhibitors and their kinetics characteristics.

Substance	IC_{50} [μM]***	Ki [μM]*	System	Ref
Metabolites of allopurinol and thiopurines				
Oxypurinol	1500**	1186	RBC	[99]
Thioxanthine	500**	0.329	RBC	[99]
Anti-inflammatory agents - 5-aminosalicylic acid derivatives				
5-ASA	1240	979	rTPTM	[95]
	129–236		RBC	[98]
Olsalazine	23	12	rTPMT	[96]
	1476	208	RBC	[97]
Sulfasalazine	78	141	rTPMT	[95]
	9–17		RBC	[97]
Balsalazide	197		rTPMT	[100]
Acethyl−5-ASA	390		rTPMT	[96]
	58–74		RBC	[97]
Immunosuppressive, Neoplastic Agents				
Methotrexate	25**		rTPMT	[101]
Non-steroidal anti-inflammatory drugs				
Naproxen	79	52	rTPMT	[98]
Mefenamic acid	39	39	rTPMT	[98]
Tolfenamic acid	63	50	rTPMT	[98]
Ketoprofen	1013	172	rTPMT	[98]
Ibuprofen	1968	1043	rTPMT	[98]
Acetylsalicylic acid		1400	*****	[51]
Diuretics				
Furosemid	15–19		RBC	[102]
		0.2	RBC	[94]
	170	0.14	rTPMT	[94]
Bendroflumethiazide	360	0.18	rTPMT	[94]
Trichlormethiazide	1000	0.27	rTPMT	[94]
Products of thiopurine methylation				
6-MMP		560		[35]
SAH		0.75****		[35]

5-ASA, 5-aminosalicylic acid; 6-MP, 6-mercaptopurine; 6-MMP, 6-methyl-mercaptopurine; RBC, red blood cells; rTPMT, recombinant thiopurine *S*-methyltransferase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine.
* In respect to 6-MP
** Approximation, according to data in the manuscript
*** In terms of TPMT activity, except for methotrexate - in terms of tryptophan fluorescence
**** With respect to SAM
***** purified human kidney TPMT

thiopurines [130,131], calls for close monitoring and dose adjustment of both drugs in certain situations.

4.2. Aminosalicylates

The aminosalicylates include 5-ASA, sulfasalazine, balsalazide, and olsalazine, and all of them are potent *in vitro* inhibitors of TPMT. These anti-inflammatory agents are used as first line treatments for mild to moderate IBD. Thiopurines have been introduced into IBD therapy for refractory disease and for lack of responsive to corticosteroid treatment [56]. Concomitant use of the thiopurines as TPMT substrates and the aminosalicylates as TPMT inhibitors is now relatively common.

4.2.1. Interactions with thiopurine S-methyltransferase

Sulfasalazine and other 5-ASA derivatives show various levels of TPMT inhibition *in vitro* [95]. While the non-competitive or mixed inhibition of recombinant TPMT by olsalazine and sulfasalazine indicate relatively strong inhibition with IC_{50} of 23 μM and 78 μM, respectively, 5-ASA and its inactive metabolite N-acetyl-5-ASA have significantly lower inhibitory effects (IC_{50} of 1240 μM and 390 μM, respectively) on recombinant TPMT (Table 2) [95–97]. Similarly, TPMT activity in erythrocyte lysates rapidly decreases with increased sulfasalazine concentration, whereas 5-ASA and N-acetyl-5-ASA have shown weaker inhibition of erythrocyte TPMT (Table 2) [97]. The bioavailability of 5-ASA and sulfasalazine suggests that the net mean plasma concentrations of these TPMT-inhibiting compounds can rise to 16 μM and 29.2 μM, respectively, during therapy [100]. According to *in vitro* studies, plasma concentrations of sulfasalazine are in the range for TPMT inhibition *in vivo* [100].

4.2.2. In silico molecular binding mode analysis

To examine the binding modes of the aminosalicylates, sulfasalazine and 5-ASA were docked to the substrate and co-factor binding sites of TPMT (mTPMT; PDB ID: 3BGD [21,44]) (Fig. 6B, SI Figure S2). Despite its lower *in vitro* inhibitory activity (Table 2), the *in silico* docking of 5-ASA to the substrate binding site of TPMT scored higher (Fred score: –9.745 kcal/mol) than that of sulfasalazine (SI Figure S2, right, red). *In silico* the 5-ASA molecule was positioned in the space of 6-MP, with its carboxylate and hydroxy groups positioned near arginine residues (Arg147, Arg221), to form ionic interactions with Arg221, and its *para*-amino group positioned near to the 'aromatic cleft' entrance that is formed of Trp24, Trp225 and Phe35. On the other hand, sulfasalazine docking to the substrate binding site of TPMT revealed a sterically constricted binding mode and consequently unfavorable scoring (Fred score: −5.022 kcal/mol) (Fig. 6B, left). The central *para*-aminobenzenesulfonamide moiety of sulfasalazine was positioned in the space of the aromatic system of the co-crystallized 6-MP (mTPMT; PDB ID: 3BGD [21,44]). The sulfonamide part of the molecule, together with the pyridine fragment, interacted with Ser34, Phe35, Trp225 and Pro190 at the 'aromatic cleft' entrance, while a diazo part and the distal carboxylic group on the salicylic fragment were positioned near to the Arg147 and Arg221 residues, respectively.

While studying the binding mode of sulfasalazine at the co-factor binding site of TPMT, favorable scores were observed (Fred score: −14.10 kcal/mol) (Fig. 6B, right). The salicylic fragment was positioned at the adenine moiety of the co-crystallized SAH (mTPMT; PDB ID: 3BGD [21,44]), and interacted with the Ser129, Ile130, Leu21 and Glu85 residues of mTPMT. The sulfasalazine molecule was oriented with its aryl-azo linker at the position of the ribosyl group on SAH, and with its pyridin-2-yl-sulfamoyl moiety out towards the distal binding pocket, which might provide interactions with the Phe35, Trp24, Gly148, Ala152, Ala68 and Arg147 residues. Here, the sulphonamide group mimicked the positioning of the sulphur atom on SAH well, marginally reaching the substrate binding cavity. The highly scored binding modes of sulfasalazine suggest that its mechanism of TPMT inhibition is directed mainly through these interactions within the co-factor binding site of TPMT, which would prevent SAM from entering the enzyme to methylate the substrate. This also explains the lower IC_{50} values of sulfasalazine compared to 5-ASA in terms of *in vitro* TPMT inhibition.

4.2.3. Clinical implications

A number of studies have addressed the rationale of concomitant use of thiopurines and aminosalicylates. The outcomes vary and the benefits have been non-conclusive. Co-treatment of thiopurines with 5-ASA in patients initially unresponsive to thiopurines resulted in elevated levels of 6-TGN [100,132–136], relatively small decreases in 6-MMP concentrations [132,134], reductions in the 6-MMP/6-TGN ratio followed by improvements of the disease parameters [132,134,137,138]. However, side effects such as myelosuppression and leucopenia were also reported [100,133,137]. Upon 5-ASA withdrawal, the 6-TGN concentrations declined to initial levels [134,136,138,139], which in certain cases resulted in relapse of IBD [138]. Recent studies reported the importance of formulations into which 5-ASA are incorporated; compared to time-dependent formulations, and matrix-dependent and pH-dependent 5-ASA formulations lead to elevated 6-TGN levels and decreased 6-MMP levels, possibly due to higher 5-ASA plasma levels which inhibit TPMT [140,141].

In standard protocols for IBD treatments, 5-ASA is chosen before sulfasalazine, and therefore there are not many clinical studies that have investigated sulfasalazine–thiopurine drug–drug interactions. Sulfasalazine might have poorer biological availability to 5-ASA; however, it has at least one order of magnitude stronger inhibition of TPMT *in vitro* [95], which suggests important perspectives for future studies.

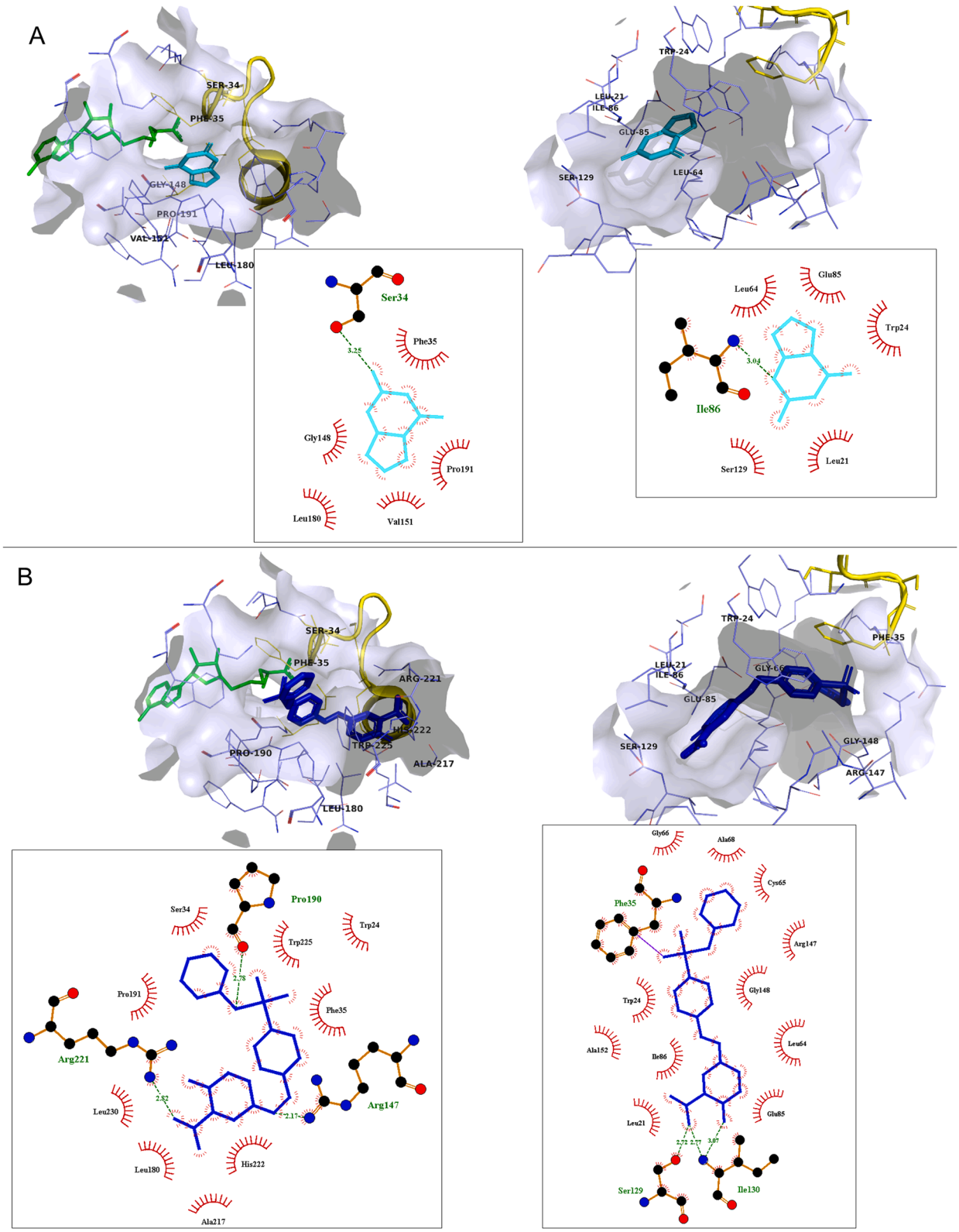

Fig. 6. A molecular docking analysis for the binding of A) oxypurinol and B) sulfasalazine to mTPMT (stick model coloured cyan and blue, respectively). Left: Binding modes of the examined molecules at the substrate binding site of mTPMT. Right: Binding modes of examined molecules at the co-factor binding site of mTPMT. Surrounding residues (PDB ID: 3BGD; [21,44]) are presented as labelled line model. Binding site surface is depicted in transparent blue-grey colour. The loop stabilized upon ligand binding [21] is coloured yellow. Below each binding mode: Respective binding mode 2D projections with measured hydrogen bonds in green, and red-emphasized hydrophobic contacts. Binding modes with superimposed SAH can be found in SI Fig. S3. PDB, protein data base; SAH, *S*–adenosylhomocysteine; (m)TPMT, (murine)thiopurine *S*–methyltransferase.

4.3. Methotrexate

Methotrexate mimics the structure of the folates, and as such it is important in the treatment of ALL [18,20] and various autoimmune disorders [142]. The combination of methotrexate with 6-MP represents a standard 'backbone' in the maintenance phase of treatment for patients with ALL [18,20,143,144]. Their use in the consolidation phase as co-therapy with other immunosuppressive and chemotherapeutic drugs can also improve outcomes for patients with intermediate-risk or high-risk ALL, and it is also beneficial for standard-risk patients [20,144,145].

4.3.1. Interactions with thiopurine S-methyltransferase and in silico molecular binding mode analysis

The effects of methotrexate on TPMT have been demonstrated in ALL patients receiving high doses of methotrexate in which methotrexate significantly decreased TPMT activities and 6-TGN levels [101,146]. Similarly, long-term *in vitro* incubations of leukemia cells with methotrexate show reduced TPMT activity, which was assumed to be through the inhibition by methotrexate (IC_{50} 25 μM; Table 2, Fig. 5) [101,146].

To delineate the methotrexate-TPMT interaction, we analyzed the binding modes of methotrexate in the substrate binding and co-factor binding sites of mTPMT (PDB ID: 3BGD [21,44]), as illustrated in Fig. 7A. After examining both of these sites, it was seen that the methotrexate scores were relatively unfavorable for the substrate binding site of TPMT (Fred score: −4.78 kcal/mol), which ranked methotrexate as the lowest relative to other compounds studied here. In contrast, methotrexate was one of the top ranked compounds in terms of a favorable docking score for the co-factor binding site of TPMT (Fred score: −12.74 kcal/mol). Methotrexate docked into the substrate binding site was positioned such that its central *para*-aminobenzoic moiety occupied the space of the aromatic system of the co-crystallized 6-MP (mTPMT; PDB ID: 3BGD [21,44]) (Fig. 7A; left). Its pteridine-2, 4-diamine was positioned near to the previously described 'aromatic cleft,' thus stabilizing the pose with H-bonds with the His222, Pro190, and Lys27 residues of mTPMT. The aminomethyl fragment of methotrexate was positioned towards the sulphur atom of SAH, and its L-glutamic acid moiety towards the distal site of the cavity towards Arg147 and Arg221 residues. Methotrexate occupied a U-shaped constricted conformation that fit sterically to the substrate binding site of TPMT. Meanwhile, methotrexate docked to the co-factor binding site of TPMT mimiced the conformation of the co-crystallized SAH (Fig. 7A, right). The pteridine-2,4-diamine part of methotrexate was positioned at the adenine moiety of SAH, to interact with the Ile86, Ser129, Ile130, and Trp24 residues of mTPMT, while the *para*-aminobenzoic moiety was located at the central ribose moiety of the co-crystallized SAH (mTPMT; PDB ID: 3BGD [21,44]). Finally, the terminal L-glutamic acid moiety of methotrexate was positioned analogously to the homocysteine part of SAH, to interact with the His36, Lys67, Gly66, Ala68, and Trp145 residues of mTPMT. Based on these findings, the binding modes of methotrexate to both the substrate and co-factor binding sites can be postulated, to encourage future studies of the methotrexate structural binding characteristics.

4.3.2. Clinical implications

Methotrexate and thiopurines have been used in the maintenance phase of ALL treatments, with the aim to reduce minimal residual disease and to improve long-term remission [143,147,148]. Longer durations of treatments and tailored doses of methotrexate and 6-MP to the limits of tolerance have been associated with better clinical outcomes [144] and reduced risk of relapse [149]. Extensive use of high-dose methotrexate with the co-administration of 6-MP have contributed to the higher cure rates of previously poorly managed adult ALL [20].

Methotrexate and 6-MP together encompass a wide range of immunosuppressive actions, which include inhibition of *de novo* purine and pyrimidine synthesis, DNA breakage, apoptosis via Rac1, and ATIC inhibition. The inclusion of methotrexate and 6-MP in a variety of processes means that each can potentiate the effects of the other, although their complete combined mechanism remains unknown, which probably reflects the complexity of their synergistic behaviors. Intracellularly, methotrexate is converted to methotrexate–polyglutamates, which are potent and stable inhibitors of the enzymes in the folate pathway and *de novo* purine synthesis. Methotrexate–polyglutamates in erythrocytes negatively influence the erythrocyte 6-MMP levels [150]. Higher methotrexate–polyglutamates concentrations also correlate with higher 6-TGN concentrations in erythrocytes [151]. The plasma concentrations of methotrexate–polyglutamates further indicate significant interactions between methotrexate and 6-MP, whereby methotrexate increases plasma concentration and the area under the curve by 6-MP [152,153], although correlations between therapeutic response and plasma 6-MP and methotrexate levels have often been contradictory [154–156]. Other synergistic mechanisms are possible for these drug–drug interactions. These include interference of methotrexate in the methylation capacity of cells via the SAM concentrations [157–159], through which methotrexate might regulate TPMT activity (Fig. 5) [7,41]. A recent study and a review paper suggest an important inhibition of TPMT by methotrexate, which may lead to higher 6-TGN levels [101,146].

4.4. Thiazide diuretics

In vitro and *ex vivo* studies have classified the thiazide diuretic furosemide as a very potent inhibitor of TPMT [94,102] (Table 2). It is listed among the first 20 most commonly prescribed drugs in the US over the last decade, with almost 30 million prescriptions per year [160]. Therefore, it is likely for furosemide to also have an impact on patients treated with thiopurines [94,161]. Furosemide is a mixed or non-competitive TPMT inhibitor. A study on erythrocyte lysates reported a furosemide IC_{50} for TPMT inhibition of 15 μM to 19 μM, with an average IC_{50} on recombinant TPMT of 170 μM [94,102]. Therapeutic plasma concentrations of furosemide are between 3 μM and 303 μM [161], and are thus in the range for TPMT inhibition.

Molecular docking analysis revealed that furosemide produced one dominant binding mode in the substrate binding site of TPMT (Fig. 7B). The binding mode was favorably scored (Fred score: −12.50 kcal/mol) and ranked as the highest relative to the other compounds studied. Furosemide consistently showed its benzene central moiety occupying the space of the co-crystallized aromatic system of 6-MP (mTPMT; PDB ID: 3BGD [21,44]). As for the binding of the co-crystallized substrate, furosemide was similarly positioned between the key aromatic residues that form an 'aromatic cleft' on one side of the substrate binding pocket: Trp24 and Phe35. In contrast to what was seen for the co-crystallized substrate (mTPMT; PDB ID: 3BGD [21,44]), furosemide effectively occupied a larger volume that spanned from the 'aromatic cleft' to the distal binding pocket that is defined by Arg147 on one side, and Leu180 and Glu232 on the other. Moreover, there were important hydrogen bonds formed between the furosemide carboxylic group and mTPMT Arg221 and Ser34 (Fig. 7B), with additional hydrogen bonds from the furosemide sulfonamide moiety towards Lys27 of TPMT. As expected, furosemide effectively interacted with the TPMT substrate binding site, with π–π stacking and multiple hydrogen bonds, but without interfering with the key catalytic sulphur atom on the co-crystallized SAH. The scores for the binding modes thus complemented the previous kinetics data of strong (competitive) TPMT inhibition, making furosemide an effective TPMT inhibitor.

In contrast to studies *in vitro*, chronic administration of furosemide increased TPMT activity in erythrocytes in renal transplant recipients and elderly patients [162]. To further reveal the background mechanisms, *in vitro* studies that reflect acute exposure of TPMT to furosemide should be translated into the *in vivo* situation.

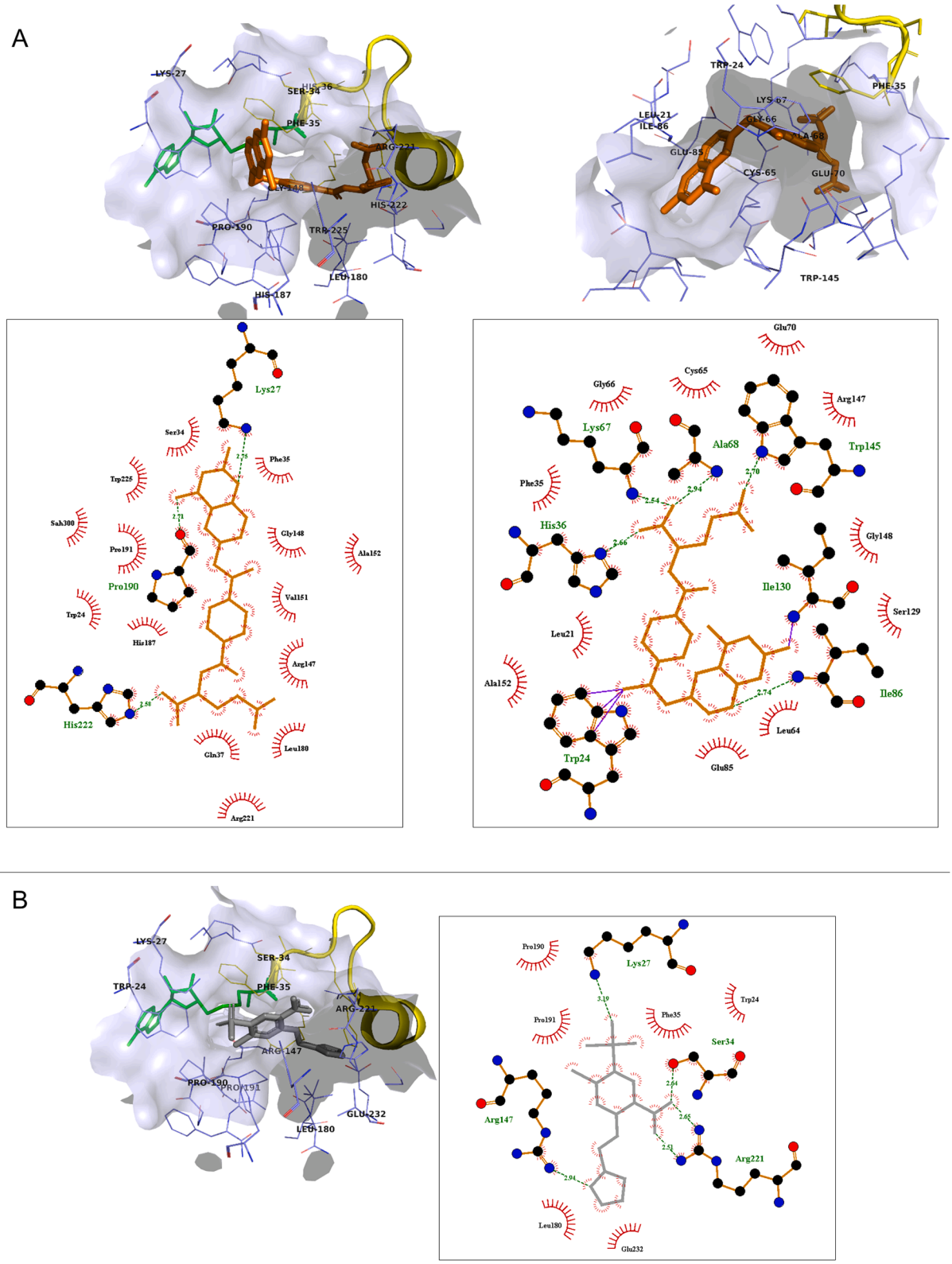

Fig. 7. A molecular docking analysis for the binding of A) methotrexate and B) furosemide to mTPMT (stick model coloured orange and grey, respectively). A) Left and B): Binding modes of the examined molecules at the substrate binding site of mTPMT. A) Right: Binding mode of methotrexate at the co-factor binding site of mTPMT. Surrounding residues (PDB ID: 3BGD; [21,44]) are presented as labelled line model. Binding site surface is depicted in transparent blue-grey colour. The loop stabilized upon ligand binding [21] is coloured yellow. Below/beside each binding mode**:** Respective binding mode 2D projections with measured hydrogen bonds in green, and red-emphasized hydrophobic contacts. Binding modes with superimposed SAH can be found in SI Fig. S3. PDB, protein data base; SAH, *S*-adenosyl homocysteine; (m)TPMT, (murine)thiopurine *S*–methyltransferase.

4.5. Non-steroidal anti-inflammatory drugs

Among other interactions reported, the most notable was *in vitro* TPMT inhibition with NSAIDs. Naproxen was shown to inhibit TPMT in a non-competitive manner with a relatively potent Ki of 52 μM, and a relatively low IC_{50} of 79 μM [98]. Given that the therapeutic concentrations that this cyclooxygenase inhibitor can reach plasma peak and steady-state concentrations between 60 μM and 70 μM, this naproxen IC_{50} implies clinical importance for *in vivo* TPMT inhibition [163]. Among other NSAIDs investigated, only mefenamic and tolfenamic acids have shown similar inhibitory potential against TPMT (Table 2). Many other common NSAIDs, such as ibuprofen, ketoprofen, paracetamol,

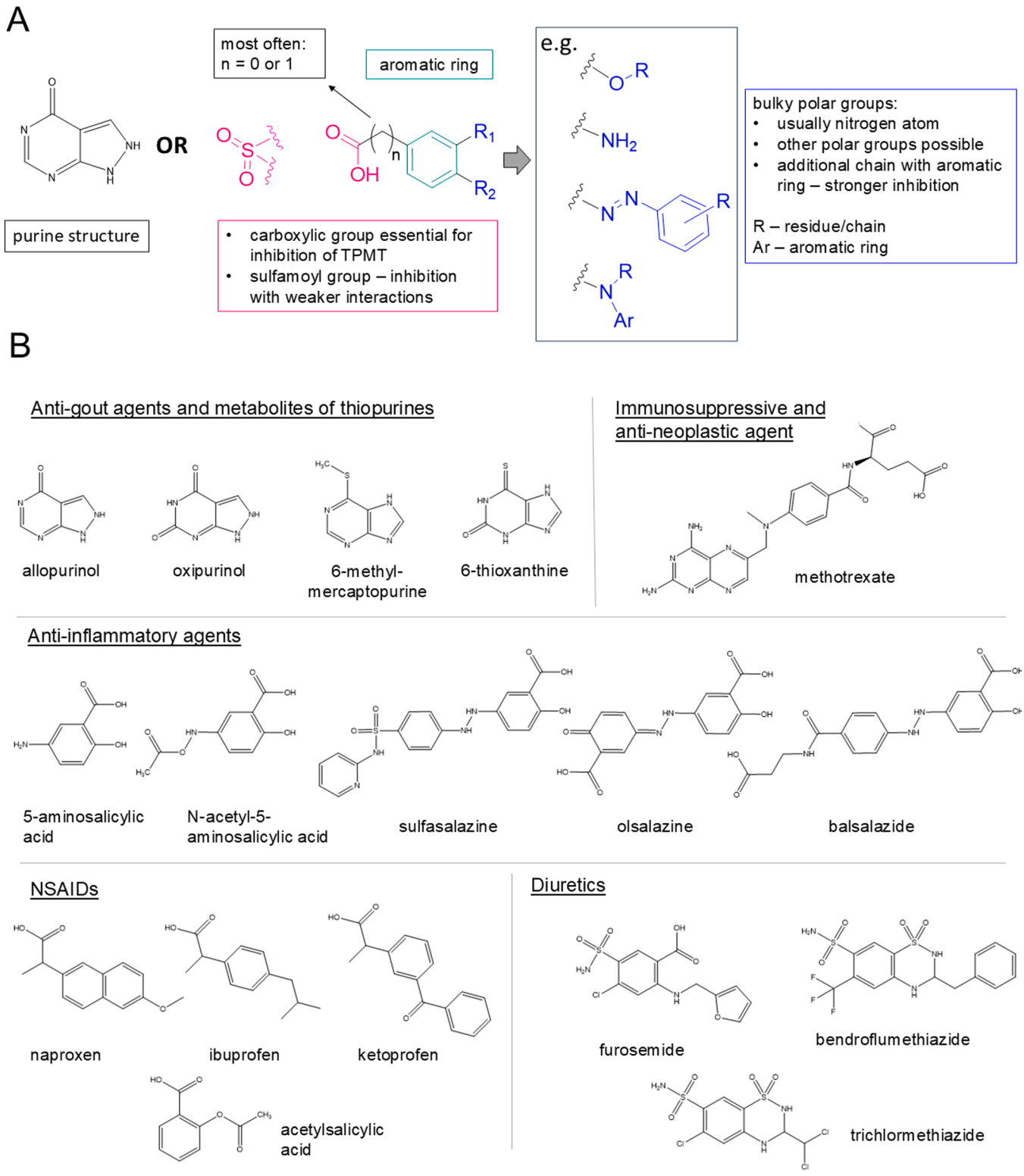

Fig. 8. Schematic representations of TPMT inhibitors. A) Structural characteristics of TPMT inhibitors. B) Chemical structures of drugs interacting and inhibiting TPMT. TPMT, thiopurine *S*-methyltransferase.

diclofenac, celecoxib, and acetylsalicylic acid have sub-clinical inhibitory effects that might relate to drug–drug interactions [98], which indicates their safer use in combination with thiopurines, compared to naproxen.

4.6. Structural characteristics of thiopurine S-methyltransferase inhibitors

All of the inhibitors of TPMT that have been reported to date are aromatic or heteroaromatic compounds (Fig. 8). The most likely binding locations for TPMT activity-modifying agents are the substrate and/or the co-factor binding sites. However, based on kinetics studies, which propose non-competitive and mixed kinetics for 6-MP and uncompetitive or non-competitive kinetics for SAM [95,97–99], other binding sites are also possible. The molecular modes of action of inhibitors involve aromatic π–π interactions with the exposed aromatic residues Phe40, Trp24, and Trp225, which are within the hTPMT and mTPMT binding sites for SAM and 6-MP [11,21]. The proposed binding sites and orientations of the purine analogues, such as oxypurinol and thioxanthine, might resemble those of the thiopurines, as their structures are very similar.

Early studies reported that a carboxylic group on the aromatic ring is essential for inhibition of TPMT [6]. All of the non-purine inhibitors that show good inhibitory kinetics do indeed have carboxylic functionality attached to the benzene ring. However, as can be seen from the naproxen molecule, this need not be attached to an aromatic ring, and can instead be separated from the ring by at least one carbon atom, while still achieving sufficient inhibition [98]. The substitution of the carboxylic group for a sulfamoyl group might lead to weaker interactions with TPMT, as the lack of this functionality in certain thiazide diuretics lowers their inhibitory potential (IC_{50} values increase from 170 μM for furosemide to 1000 μM for trichlormethiazide) [94]. The importance of the -COOH group is also evident for sulfapyridine, a molecule that is formed after bacterial degradation of sulfasalazine by the cleavage of the diazo bond. Sulfapyridine does not contain a carboxylic group, and it has no effect on the activity of TPMT [95].

Another important and previously described determinant that can enhance TPMT inhibition is *para* and *meta* substitution of the aromatic ring with bulky polar groups [51]. In almost all cases of the aromatic substances studied, the substituents attached to the benzene ring are positioned *para* or *meta* with the nitrogen atom, as a link between the ring and the following chain. Both of these characteristics can be estimated by comparing different 5-ASA analogues. Sulfasalazine is 15-fold more active compared to 5-ASA alone in terms of IC_{50} (75 μM vs. 1240 μM, respectively) [95]. Even stronger inhibition of TPMT can be achieved by olsalazine [96]. In contrast to the simple structure of 5-ASA, its diazo analogues contain a larger *meta*-positioned chain with at least one additional aromatic ring. This part of the molecule appears to form additional hydrophobic/aromatic interactions with TPMT and has positive stearic characteristics that stabilize the orientation of the molecule and that prevent SAM or the substrate from entering the active site. Similarly, the polar part of the *para*- or *meta*-positioned side chain seems to play a significant role also in TPMT inhibition by NSAIDs. Naproxen and ketoprofen, which contain hydroxyl or carbonyl groups in their side chains, respectively, exhibit stronger inhibitory effects on TPMT compared to ibuprofen, which lacks a polar group in its side chain, or amino salicylic acid, which has an *ortho*-substituted polar acetic ester. Notably, naproxen is the strongest inhibitor among the NSAIDs investigated, suggesting that the naphthalene ring may contribute to additional aromatic interactions with TPMT, enhancing its inhibitory potency.

5. Conclusion

Adverse drug reactions are a global health problem and account for 3.5 % of hospital admissions in Europe [164]. Thiopurines continue to serve as a cornerstone in the management of ALL and IBD despite the advancements in available treatment options and the fact that adverse effects are observed in up to a third of patients [148,165]. The activity of the TPMT enzyme has long been acknowledged as playing a crucial role in determining the outcome of thiopurine treatment, especially in relation to adverse drug reactions. Understanding the molecular mechanisms and interactions involving TPMT is essential for the development of new therapeutic strategies, the optimization of existing treatments and the prediction and management of drug toxicities [119,140,141,146,166].

The aim of this review was to provide an in-depth analysis of TPMT as an important intersection in drug-drug interactions in thiopurine treatment. First, we explored the binding sites for substrates and co-factors augmented by molecular docking analysis and presented new insights into the endogenous function of the TPMT gained in recent years [8,10,50,90]. Beside thiopurines, their derivatives and selenocysteine, several synthetic compounds including thiophenols, selenopurines, and 2-thiouracyl have been identified as substrates of TPMT. We then focused on the principal interactions of TPMT with clinically significant inhibitors, supplementing this with molecular docking analyses. When studying inhibition of TPMT, inhibitors appeared to bind most likely to the substrate and/or to the co-factor binding sites. Our *in silico* docking analysis of the most relevant TPMT inhibitors showed that smaller molecules, such as furosemide and aminosalicylic acid effectively occupy substrate binding site, whereas larger molecules, such as sulfasalazine and methotrexate bind most favorably to the co-factor binding site.

Drug-interaction studies of TPMT inhibitors have resulted in the translation of biochemically-based findings into clinical practice for optimization of the thiopurine therapy. First, a co-therapy of thiopurines and allopurinol in IBD, followed by implementations also in ALL and hepatic autoimmune diseases, have opened the possibility to overcome refractory events, as well as the severe side effects of thiopurine therapies. Next, with the established methotrexate–6-MP co-therapy in ALL, children can now experience long-term event-free survival. However, the benefits of the concomitant use of thiopurines and aminosalicylates have not been conclusive. Based on reviewed studies, several previously overlooked and hidden interactions can be envisaged that trigger toxic side effects of thiopurines. Given the use of thiopurines in the treatment of ALL, IBD, and autoimmune disorders, it is critical to acknowledge these hidden risks. Specifically, when thiopurines are prescribed alongside medications like naproxen for pain management or furosemide for the maintenance of normal blood pressure, the likelihood of harmful interactions increases. These drugs, which can alter the pharmacokinetics of thiopurines, may amplify the risk of toxicity, making it essential for healthcare providers to monitor patients more closely.

Future research could focus on evaluating the clinical importance of the identified TPMT inhibitors in patients carrying different *TPMT* genotypes as well as explore genetic *TPMT* variants across diverse populations. Additionally, exploring alternative pain management strategies or blood pressure maintenance treatments for patients on thiopurines may offer safer therapeutic options.

Abbreviations

5-ASA, 5-aminosalicylic acid; 6MP, 6-mercaptopurine; 6MMP, 6-methylmercaptopurine; 6 TG, 6-thioguanine; 6TGN, 6-thioguanosine nucleotides; ALL, acute lymphoblastic leukemia; AZA, azathioprine; CPIC, Clinical Pharmacogenetics Implementation Consortium; DPWG, Dutch Pharmacogenetics Working Group; GDA, guanine deaminase; GMPS, guanosine monophosphate synthase; GST, glutathione transferase; HGPRT, hypoxanthine phosphoribosyl transferase; IBD, inflammatory bowel disease; IC, inhibitory concentration; IMPDH, inosine monophosphate dehydrogenase; NSAID, non-steroidal anti-inflammatory drug; PDB, protein data bank; RMSD, root mean square deviation; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TPMT, thiopurine S-methyltransferase; TYMS, thymidylate synthase; VNTR,

variable number of tandem repeats; XO, xanthine oxidase.

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Data statement

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CRediT authorship contribution statement

Urbančič Dunja: Writing – original draft, Data curation, Conceptualization. **Jukič Marko:** Writing – original draft, Data curation. **Šmid Alenka:** Writing – original draft, Data curation. **Gobec Stanislav:** Writing – review & editing, Funding acquisition. **Jazbec Janez:** Writing – review & editing, Data curation. **Mlinarič-Raščan Irena:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.117893.

Data Availability

We have shared all the information on data used for the MD analysis in the main manuscript and supplementary material.

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