

Chapter 2

Selectivity of TPMT towards thiopurine nucleosides and nucleotides

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Abstract

Detoxification of xenobiotic compounds is a common defense mechanism to prevent the accumulation of toxic compounds. Thiopurine-S-methyltransferase (TPMT) is one of the methyltransferases involved in methylation of thiol containing heterocyclic compounds, e.g. the thiopurines. Thiopurines are widely used in the treatment of inflammatory disorders and polymorphism in thiopurine metabolizing enzymes like TPMT cause serious adverse events.

To explore the methylation of the various thiopurinebases, -nucleosides and -nucleotides by TPMT, incubation studies were performed. Enzyme sources were recombinant human TPMT, erythrocyte lysates and cell cultures of MOLT-3 cells. As methyl donor both unlabeled and tritium labeled S-adenosyl methionine (SAM) were used in these studies.

Results of these studies showed that, as expected, 6-mercaptopurine and 6-thioguanine were methylated by all enzyme sources. 6-thioinosine-5'-monophosphate on the other hand was methylated by hrTPMT, but not in erythrocyte lysates. In MOLT-3 cultures we detected MTIMP after incubation with 6-MP. Incubation of MOLT-3 cells with 6-TG clearly showed the formation of the consecutive thioguanine nucleotides. Only a modest amount of MTGMP was present. Incubation with 6-MMP showed no formation of 6-MTIMP, hereby excluding the phosphoribosylation of 6-MMP by HGPRT.

Studies on the methylation of thiopurinucleotides in cells from patients with different defects in (thio)purine metabolizing enzymes or with specific inhibition of pathways are needed to further elucidate the way thiopurines are metabolized.

Introduction

Purines and pyrimidines are heterocyclic compounds essential for mammalian life: precursors of RNA and DNA, involved in signal transduction, essential co-factors in enzyme reactions and other indispensable functions. Purine metabolism is a complex network of enzymes catalyzing *de novo* synthesis, salvage, degradation and interconversion of purines bases, nucleosides and nucleotides. Defects in enzymes involved in this network result in inborn errors of metabolism with a broad spectrum of clinical symptoms and metabolic disturbances¹. So far defects in all purine metabolizing pathways are described and are detectable by metabolite analysis, enzyme activity measurement, mutation analysis or combinations of these techniques. Deactivation of exogenous metabolites is an important *in vivo* defense mechanism to control the concentrations of potentially toxic compounds. Thiopurines, substituted analogs of purines, are widely used as antimetabolites for the treatment of leukemia, organ rejection and inflammatory disorders². Their efficacy and safety depends highly on the routes involved in the activation, degradation and deactivation of these compounds³. Thiopurines are activated and metabolized by the enzymes of the degradation, salvage and interconversion routes of purine metabolism. In addition thiopurine bases are deactivated by the enzyme thiopurine S-methyltransferase (TPMT)^{4,5}. In replicating cells, a high TPMT activity will result in a reduced availability of the activated metabolite 6-thioinosine-5'-monophosphate (TIMP) for the interconversion to the active compounds, the thioguanine nucleotides, and in higher concentrations of therapeutically inactive, but nevertheless toxic, methylthiopurine-nucleotides⁶. Reduced TPMT activity will increase the concentrations of thioguanine nucleotides to toxic limits in replicating cells⁷.

The only recognized role of TPMT in mammalian metabolism is to deactivate thiol containing purine (like) compounds into less active, and supposedly less toxic, metabolites⁸. In aqueous ecosystems TPMT is involved in the detoxification of selenium-containing compounds. Whether TPMT is involved in selenium metabolism in man is unclear, although the high affinity for selenium containing purines suggests an active role of the enzyme in this context⁹.

TPMT is thought to be involved not only in the deactivation of thiopurine bases, but also in the methylation of thiopurine nucleosides and nucleotides. Although this is generally accepted, little is known about the selectivity of TPMT towards the thiopurine-nucleosides and -nucleotides¹⁰. The limited studies on this subject report the substrate specificity of these compounds for human TPMT (hTPMT), which was either purified from human tissue or obtained by a yeast expression system^{10,11}. It is apparent that this presumed selectivity for thiopurinenucleotides will influence the bio-availability of these compounds and hence efficacy of the therapy.

To obtain more insight in the role of TPMT in deactivation of thiopurine metabolites a number of experiments were started. First the substrate specificity of hTPMT towards different thiopurine bases, nucleosides and nucleotides was characterized, using

recombinant human TPMT (rhTPMT) and hTPMT from lysed erythrocytes as the enzyme source. Experiments were repeated with loading of cultured MOLT-3 cells during a fixed period with thiopurines. (Methyl)Thiopurine nucleotides were measured using HPLC-UV detection, with the aid of the appropriate separation conditions.

Materials and methods

Recombinant human TPMT from an *E. coli* expression system, full length protein (245 aa), was obtained from Abcam (Cambridge, United Kingdom). Thiopurine bases, nucleosides and nucleotides were obtained from Sigma (Zwijndrecht, the Netherlands) or from Jena Bioscience (Jena, Germany). Methylated thiopurine-bases, - nucleosides and -nucleotides and ^{14}C -6-mercaptopurine were obtained from Jena Bioscience (Jena, Germany). Adenosyl Methionine,S-(methyl ^3H) (^3H -SAM) was purchased from Perkin Elmer (Waltham, MA, USA). Ultra-pure acetonitril was purchased from Biosolve (Valkenswaard, the Netherlands) All other chemicals were of the highest quality and purchased from Sigma (Zwijndrecht, the Netherlands).

Incubation experiments

Conditions for incubations of the different substrates with hrTPMT were identical to the conditions used for the measurement of TMPT activity in lysed red blood cells: samples were preincubated with the substrate. After 3 minutes the methyl donor SAM or ^3H -SAM was added and the incubation prolonged to 1 hr. The reaction was stopped with ice-cold 10% HClO_4 . After neutralization the sample was ready for HPLC separation. Molt-3 cells were cultured and incubated with the different substrates and (un)labeled SAM during 4 and 24 hrs. Cells were washed with medium and isolated. Intracellular thionucleotides were measured in perchloric acid extracts.

Separation of (methyl)thiopurine bases, nucleosides and nucleotides

Separation of 6-TG from 6-MTG (AMMP) was achieved using a reversed-phase Nucleosil 100-5-C18 column, 250 mm \times 4.6 mm, particle size 5 μm (Bischoff Chromatography, Leonberg, Germany). The HPLC system (Shimadzu, 's-Hertogenbosch, The Netherlands) consisted of a system controller (SCL-10A), a binary pump (LC-10AD), an autosampler (SIL-10AD) and a DAD detector (SPD-M10A). The mobile phase consisted of 0.1% acetic acid (Solvent A) and 100% acetonitrile (solvent B). The components were separated using the following gradient: 0 to 15.0 min, 88% A; 20.0 to 25.0 min, 50% A; 29.0 to 35.0 min 88% , flow rate 1.0 ml/min. Column temperature: 21°C. 50 μl sample was injected into the HPLC system and AMMP was detected at 290 nm. For the detection of the radioactive labeled compounds a flow scintillation

analyzer (FSA) Radiomatic 625 TR (PerkinElmer, Groningen, the Netherlands) was coupled in series to the HPLC system.

6-MP / 6-MMP, 6-TIMP / 6-MTIMP and 6-TGMP / 6-MTGMP were analyzed as described before¹².

Separation of (methyl)thiopurinenucleotide di- and triphosphates was performed on a PerkinElmer Series 200 HPLC system (PerkinElmer, Groningen, the Netherlands) Separation was achieved using a PartiSphere SAX column (4.6 x 125 mm, 5 μ m, Whatman International Ltd, Maidstone, United Kingdom) protected by the appropriate guard cartridge and maintained at 21°C. The mobile phase consisted of 9 mM ammonium dihydrogen phosphate, pH 3.5 (solvent A) and 325 mM ammonium dihydrogen phosphate and 500 mM potassium chloride, pH 4.4 (solution B). Separation was achieved using the following gradient: 0 to 2 min, 100% A; 2 to 12 min, 20% A; 12 to 17 min, 10% A; 17 to 47 min, 10% A; 47 to 48 min, 100% A; 48 to 58 min, 100% A. The flow rate was 1.0 ml/min; injection volume 50 μ l. The detector was set at the maximal absorbance wavelength for 6-TIMP and 6-MTIMP, 322 nm and 292 nm respectively. 6-TGTP and 6-MTGTP were detected at 341 nm and 309 nm respectively. Data acquisition and handling was performed by using Totalchrom software (Perkin-Elmer, Groningen, the Netherlands) except for the quantification of 6-TG and 6-AMMP which was performed by using Class-VP software (Shimadzu, 's-Hertogenbosch, the Netherlands). External standards with known concentrations of the components of interest were used to determine the retention times of the different metabolites and to calculate the activity of TPMT by relative peak areas. All experiments were performed in duplicate.

Results

From the results of the incubation experiments of various thiopurine derivatives with rhTPMT we confirmed that 6-MP and 6-TG were substrates for the enzyme, the calculated kinetic parameters were in line with earlier published values (Table 2.1).

Table 2.1 Kinetic properties of hrTPMT for 6-MP and 6-TG.

Substrate					
6-MP		6-TG		ref	
Km (μ M)	Vmax (nmol/mg/min)	Km (μ M)	Vmax (nmol/mg/min)		
323	2.32	603	1.72	Present study	
320	n.d.	200	n.d.	[13]	
300	n.d.	550	n.d.	[11]	
383 \pm 7	14.95 \pm 0.15	557 \pm 10	18.0 \pm 0.3	[9]	
10.6 \pm 1.3	48 \pm 3	18.1 \pm 3.4	55 \pm 5	[10]	
		34.9 \pm 6.7	122.8 \pm 16.9	[14]	
		15.2 \pm 0.3	19.2 \pm 1.0	[15]	

The results of the incubation experiments with the other thiopurine derivatives, e.g. 6-thioinosine and 6-TIMP were not consistent and we repeated the incubations with tritium labeled SAM as the methyl donor. We could confirm transfer of the tritium label to the different substrates (Table 2.2). The compounds were separated with HPLC and the retention times were confirmed by non-labeled standards, detected by UV detection, coupled in series with a flow scintillation analyzer.

Table 2.2 Incorporation of ^3H -methyl label in thiopurine compounds by RHTPMT.

Compound	Product	Label incorporated (%)	
		6 hr	24 hr
6-MP	6-MMP	10.2 ± 0.40	15.3 ± 0.95
6-TG	6-MTG	8.9 ± 0.25	16.7 ± 1.55
TIMP	6-MTIMP	11.3 ± 0.64	16.4 ± 0.57
6-TI	6-MTI	11.4 ± 0.64	16.3 ± 0.62

We repeated this experiment by incubating MOLT-3 cells over 24 hours with 6-TI, 6-MP and 6-MMP, together with tritium labeled SAM. Incubation with 6-TI showed formation of 6-MP and 6-MTIMP, the latter compound could be detected through the transfer of the ^3H -methyl group from SAM. Incubation with ^{14}C -6-MP resulted in the formation of 6-MTIMP, both the ^3H and ^{14}C label were detected at the retention time of 6-MTIMP, 6-MMP was not found in the intracellular extracts. From the incubation experiment with 6-MMP we could confirm that 6-MMP enters the cell and stays unchanged. No changes in the pattern of intracellular thiopurine metabolites were detected.

When a full (methyl)thionucleotides profile was analyzed, 4 hours after addition of 6-MP to the cell culture 6-TIMP and 6-MTIMP were detectable. Using 6-TG as a substrate 6-TGMP, 6-TGDP and 6-TGTP (ratio 12:4:1) were detectable after 4 hours of incubation (Figure 2.1). Only a modest amount of 6-MTGMP was detectable after 4 hours.

We also tested human erythrocyte lysates with the different substrates: we could only confirm the S-methylation of 6-MP and 6-TG, 6-TIMP was not methylated. In theory MTIMP can be generated from the phosphoribosylation of 6-MMP by HGPRT. To test this hypothesis we incubated 6-MP and 6-MMP with PRPP in an erythrocyte lysate under conditions used for HGPRT activity measurement. As expected 6-MP was converted to TIMP, but with 6-MMP as substrate no product was generated.

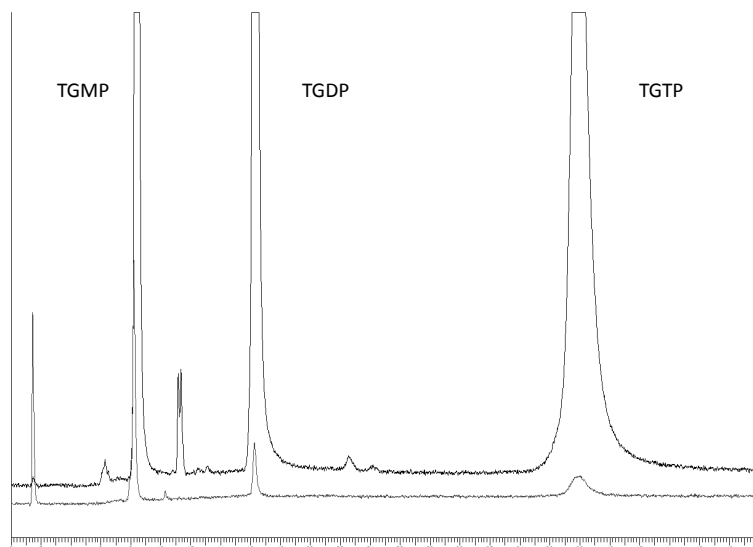


Figure 2.1 Thionucleotide profile in molt-3 extracts after loading with 6-TG (lower trace); upper trace standards TGMP, TGDP, TGTP Rrespectively. Chromatographic conditions are described in the methods section.

Discussion

The ways thiopurines exert their effect is still not fully understood. The original idea of using thiopurines as drugs in cancer therapy was that these compounds would act as purine antimetabolites through the incorporation of (deoxy)thiopurinenucleotides of guanine into RNA and DNA, thereby inhibiting further replication. A second effect which was observed was the inhibition of the purine *de novo* synthesis (PNDS) by the methylated thiopurinenucleotides formed *in vivo*. Methyl-thioinosine-monophosphate (MTIMP) was identified as the inhibitory compound, although earlier reports also named methylthioinosine as the responsible metabolite to inhibit PNDS^{16,17}. In general it is assumed that MTIMP is generated by the action of TPMT on TIMP. Our experiments with rhTPMT and labelled ³H-SAM show that MTIMP is synthesised *in vitro* from TIMP. We could also confirm the formation of MTIMP in MOLT-3 cells after incubation with 6-MP. In erythrocyte lysates no methylation of TIMP occurred. The reason why TIMP was not methylated in erythrocyte lysates remains unsolved. From the results from the incubation of rhTPMT with ³H-SAM it is clear that the transfer of the methyl group is as efficient to 6-MP as to TIMP (Table 2.2).

In intact cell systems other enzymes will play an additional role in thiopurine metabolism. Nucleated cells have high purine 5'-Nucleotidase (5-NT) activity: this

enzyme has a high affinity for TIMP, which will be degraded to thioinosine by the action of 5-NT¹⁸. As reported earlier thioinosine is a substrate for TPMT and methylthioinosine will be formed⁹. Results from experiments in endothelial cells show that methylthioinosine is readily converted to MTIMP. The authors suggest the action of adenosine kinase (AK) responsible for this conversion¹⁹. Our results also confirm methylation of 6-TI by hrTPMT (Table 2.2). Earlier studies on TPMT suggest the existence of another thiol methyltransferase, 'alkyl thiol methyltransferase'. This microsomal enzyme showed overlapping characteristics with TPMT⁸.

Currently experiments are planned to further elucidate the methylation of thiopurines. By using inhibitors of enzymes involved in (thio)purine metabolism more insight can be gained regarding the fate of thiopurines in mammalian metabolism.

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