Chapter 6

The effect of *ITPA* polymorphisms on the enzyme kinetic properties of human erythrocyte inosine triphosphatase towards its substrates ITP and 6-thio-ITP

Effects of *ITPA* polymorphism on ITPase kinetics

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Submitted

Summary

The role of inosine triphosphatase (ITPase) in adverse drug reactions associated with thiopurine therapy is still under heavy debate. Surprisingly little is known about the way thiopurines are handled by ITPase. We studied the effect of *ITPA* polymorphisms on handling of inosine triphosphate (ITP) and thioinosine triphosphate (TITP) to gain more insight in this phenomenon.

Human erythrocyte ITPase activity was measured by incubation with ITP, using established protocols and the generated IMP was measured using ion pair RP-HPLC. Molecular analysis of the *ITPA* gene was performed to establish the genotype. Kinetic parameters were established for the 2 common polymorphisms, both for ITP and TITP as substrates, using the above mentioned protocol.

Both ITP and TITP are substrates for ITPase and enzyme activities are for these substrates comparable. Substrate binding is not altered in the different *ITPA* polymorphisms. It is shown that the velocity of pyrophosphohydrolysis is compromised when the c.94C>A polymorphism is present, both in the heterozygous or homozygous state.

TITP is handled by ITPase in a similar way as ITP, which implies that TITP will accumulate in cells of patients with an ITPase deficiency, resulting in adverse drug reactions on thiopurine therapy. In carriers of *ITPA* polymorphisms the matter is more complex and the development of ADR may depend on additional, epigenetic, factors.

Introduction

Thiopurines are purine anti-metabolites, widely used as anti-inflammatory agents. These compounds are activated and degraded by the enzymes of the purine pathways for activation, interconversion and degradation. One of the enzymes involved in this metabolism is inosine triphosphatase (ITP pyrophosphohydrolase; ITPase; EC 3.6.1.19). As is shown in Figure 6.1 ITPase is part of the inosine nucleotide cycle. The exact role of ITPase in mammalian metabolism is still unclear. Primarily ITPase plays an important role in the homeostasis of non-canonical purine nucleotides, in addition it is generally considered as a house keeping gene[1,2]. Deficiency of ITPase results in the intracellular accumulation of inosine triphosphate (ITP)[3]. The clinical consequences of this phenomenon are unknown as it is not related to overt pathology[4].

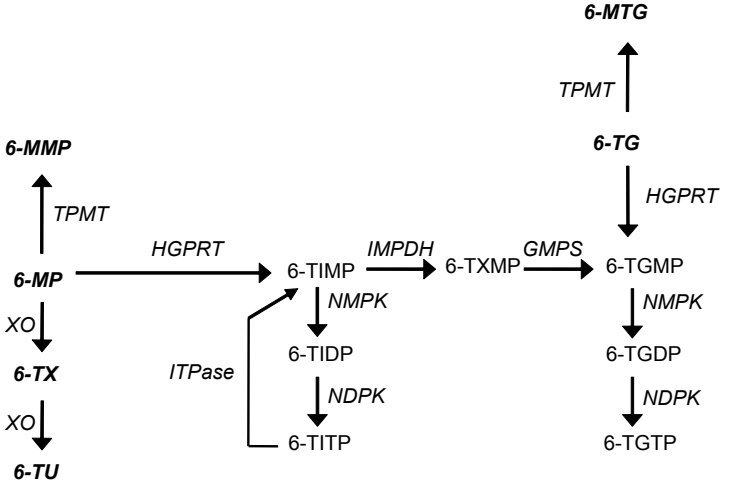

Figure 6.1 Concise scheme of thiopurine metabolism in mammals.
GMPS: Guaninemonophosphate synthase; HGPRT: Hypoxanthine-guanine phosphoribosyl transferase; IMPDH: Inosinemonophosphate dehydrogenase; ITPASE: Inosine triphosphatase; 6-MMP: 6-Methylmercaptopurine; 6-MP: 6-Mercaptopurine; 6-MTG: 6-Methylthioguanine; ND/MPK: Nucleotide mono/diphosphatase kinase; 6-TG: 6-Thioguanine; 6-TGM/D/TP: 6-Thioguanine/di/triphosphate; 6-TIM/D/TP: 6-Thioinosinemono/di/triphosphate; 6-TU: 6-Thiouric acid; 6-TX: 6-Thioxanthine; 6-TXMP: 6-Thioxanthosine-monophosphate.

We recently showed that 6-thio-inosinetriphosphate (TITP) is a substrate for human erythrocyte ITPase[1]. It is assumed that on thiopurine medication a diminished activity of ITPase leads to adverse drug reactions (ADRs), caused by the proposed undesired accumulation of TITP, and consecutively therapy failure[5]. At present the role of *ITPA* nucleotide polymorphisms in thiopurine induced ADRs is under debate and

contradicting results have been published. In patients suffering from inflammatory bowel disease (IBD), the reported ADR associated with thiopurine therapy reported are pancreatitis, flu-like symptoms, leucopenia, rash and hepatotoxicity and appear to be associated with the c.94 C>A polymorphism[5-7]. The g.IVS2+21 A>C polymorphism was reported to be associated with thrombopenia[8]. Other studies in (paediatric) IBD and renal transplant populations did not reveal any side effects correlated to *ITPA* polymorphisms, as was reported in a meta-analysis of studies on *ITPA* polymorphisms and thiopurine toxicity[9-11]. However, the populations in the above mentioned studies differ greatly and the number of patients included in each study is limited, therefore not allowing definitive conclusions on the association of *ITPA* polymorphisms and ADR[12]. Moreover, only very little mechanistic data are available on ITPase, providing insight in the binding and handling of its substrates, hereby making interpretation of the conclusions from the different studies even more difficult[13,14]. Recently the crystal structure of the enzyme was elucidated and the implications of the c.94C>A polymorphism in substrate handling predicted[15].

One of our major research topics is to elucidate the role of ITPase in thiopurine metabolism. From this perspective we studied the effect of the two common *ITPA* polymorphisms on the handling of ITP and TITP by human erythrocyte ITPase. In this study we established reference values for the different polymorphisms for the natural substrate ITP. In addition, we compared the pyrophosphohydrolysis of TITP in relation to that of ITP for these polymorphisms. For better understanding of these values the kinetic parameters for the pyrophosphohydrolysis, both for ITP and TITP, were determined for the different *ITPA* polymorphisms. This study is the first to establish TITP kinetics of human erythrocyte ITPase and the effect of polymorphisms in the *ITPA* gene on the kinetic properties of ITPase.

Materials and Methods

Materials

Recombinant human ITPase from an E. coli expression system, full length protein, 195 amino acids (#AAH10138), was obtained from Abnova (Bioconnect, Huissen, The Netherlands). Thiopurinenucleotides were obtained from Sigma (Zwijndrecht, the Netherlands) or Jena Bioscience (Jena, Germany). Ultra-pure acetonitril was purchased from Biosolve (Valkenswaard, the Netherlands). All other chemicals were of the highest grade and purchased from Sigma (Zwijndrecht, the Netherlands).
HPLC separations were performed on a Supelcosil LC-18 S column (Sigma, Zwijndrecht the Nethterlands), using an Alliance Separation system (Waters, Etten-Leur, the Netherlands) coupled to a Jasco Multi-Wavelength detector (Jasco Benelux, IJsselstein,

The Netherlands). Data were analysed with the aid of Totalchrom data acquisition and handling software (Perkin-Elmer, Groningen, the Netherlands).
Hemoglobin content of erythrocyte lysates was measured using a Coulter LH-750 hematology analyzer (Beckman Coulter, Mijdrecht, The Netherlands).

Patients

Samples of patients referred to our laboratory for preventive pharmacogenetic testing of thiopurine-S-methyltransferase (TPMT) and ITPase were used in this study. The study was performed under local ethical standards on the use of patient samples for research.

Measurement of erythrocyte ITPase activity and kinetic parameters

Assays of ITPase activity in erythrocytes were performed as described earlier[16,17]. Briefly, erythrocytes were isolated after centrifugation. Saline washed erythrocytes were lysed with ice-cold water (1:4, v/v) and the lysate was stored at -80^{o}C until ITPase measurement. Lysates were stable for >3 years and could withstand 3-4 thaw-freeze cycles before the ITPase activity dropped (data not shown). The erythrocyte lysate was incubated with ITP, $MgCl_2$ and DTT in Tris-HCl buffer (pH 8.5) for 30 minutes at 37^{o}C. The end product, inosine monophosphate (IMP), was measured using ion-pair reversed-phase chromatography with UV-detection at 254 nm[16]. Activity was calculated using an external standard method and normalised to haemoglobin (Hb) concentration. For the determination of the kinetic parameters for ITP and TITP the same method was used, with the exception of a slight modification of the solvent system and detection wavelength (320 nm) for the quantification of the thioinosine nucleotides. All kinetic experiments were performed in triplicate on three different samples per genotype.

ITPA genotyping

Genomic DNA was extracted from buffy coats using the Qiagen FlexiGene DNA kit (Qiagen, Venlo, The Netherlands) and the automated DNA isolation robot Autogenflex 3000 (Westburg, Leusden, The Netherlands).
Exon 2 of the *ITPA* gene and flanking intronic regions were amplified by PCR using primers ITPA2F-CTTTAGGAGATGGGCAGCAG and ITPA2R-CACAGAAAGTCAGGTCACAGG. PCR mix (10 µl) consisted of 1x Amplitaq Gold Mastermix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), 8% glycerol and 200 nM of each primer. PCR conditions were 40 cycli and and T_{an} of 60°C. The resulting 241 bp PCR product was bidirectional sequenced using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction kit and the ABI3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Functional polymorphisms c.94 C>A (p.P32T) (NCBI rs1127354) and g.IVS2+21 A>C (NCBI rs7270101) were determined using DNA variant analysis software Mutation

Surveyor® with genomic NCBI reference sequence NC_000020. All sequences were evaluated by two independent laboratory experts.

Results

In total, 160 patients were fully characterized for ITPase activity and *ITPA* gene polymorphisms. The distribution of the ITPase activity in erythrocyte lysate for the different genotypes is shown in Figure 6.2. From this distribution it is apparent that there is a certain overlap in the activities measured in the different polymorphisms. No overlap was observed for the c.94 CA / g.IVS2+21 AC and c.94 AA / g.IVS2+21 AA genotypes, which both had significantly lower enzyme activities ($p<0.001$ with all other genotypes).

In our cohort of patients we identified two other, so far unknown, mutations. One point mutation, c.97 T>C, is located near the frequently occurring and activity lowering c.94 C>A polymorphism. In the individual heterozygous for this new mutation, the ITPase activity is decreased to values as detected in c.94 C>A heterozygotes: 1.20 and 1.43 mmol IMP/mmol Hb/hr respectively. A second mutation, c.122 A>G, did not affect ITPase activity. A third patient had a nearly undetectable ITPase activity, but we only detected one polymorphic allele, harbouring the c.94C>A mutation. Investigations are in progress in finding a possible second molecular aberration in the *ITPA* gene to explain the low ITPase activity in this patient.

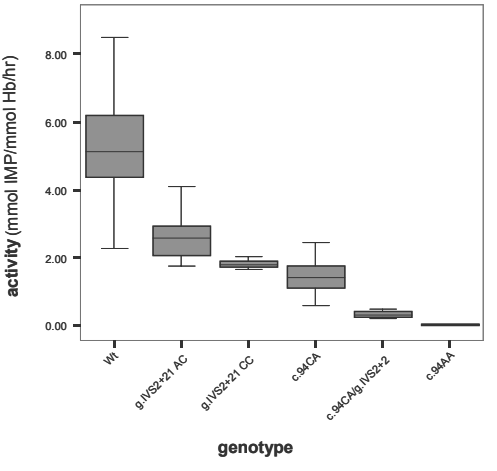

Figure 6.2 ITPase activity (mmol IMP/mmol Hb/hr) in different *ITPA* genotypes.

In Table 6.1 the correlation between ITPase activity and the most common activity lowering polymorphisms is displayed, compared with the relative activities published

in the literature in the past years. As can be seen, our present results using ITP as the substrate are in line with those of other authors.

To investigate the selectivity of the different *ITPA* polymorphisms for the substrates ITP and TITP the kinetic properties V_{max} and apparent K_m (further referred to as K_m) for these substrates were determined. The results of these experiments are shown in Table 6.2. The genotypes tested were the wild type, the compound heterozygous c.94 C>A/g.IVS2+21 A>C variant , the homozygous g.IVS2+21 A>C and the heterozygous c.94 C>A and g.IVS2+21 C>A genotypes. Because of the very low residual activity of the c.94 C>A homozygote the experiment was not performed for this genotype.

Table 6.1 Erythrocyte ITPase activity (mmol IMP/mmol Hb/hr) for the different *ITPA* genotypes, compared with relative activities derived from the literature.

		Present study		Literature		
ITPA genotypes	N	Mean ± SD	%WT	[17]	[18]	[19]
c.94 CC /g.IVS2+21 AA	88	5.27 ± 1.56	100	100	100	100
c.94 CC / g.IVS2+21 AC	19	2.61 ± 0.66	49.5	58.6	61.0	
c.94 CA /g.IVS2+21 AA	39	1.43 ± 056	27.1	22.5	25.5	27.3
c.94 CC / g.IVS2+21 CC	3	1.81 ± 0.19	34.4		29.8	
c.94 CA / g.IVS2+21 AC	6	0.32 ± 0.10	6.1	9.0	8.2	
c.94 AA / g.IVS2+21 AA	5	0.00 ± 0.05	0.0		0.1	0.0

Table 6.2 Kinetic parameters for ITP and TITP for different *ITPA* polymorphisms.

ITPA genotypes	ITP			TITP		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
c.94 CC / g.IVS2+21 AA	201	5.44	27.1	346	5.95	17.2
c.94 CA / g.IVS2+21 AA	162	0.98	6.1	313	1.43	4.5
c.94 CC / g.IVS2+21 AC	144	4.0	27.4	576	2.82	4.9
c.94 CA / g.IVS2+21 AC	366	0.43	1.2	219	0.41	1.9
c.94 CC / g.IVS2+21 CC	170	1.54	9.1	164	1.73	10.5

K_m : µM ITP or TITP; V_{max}: mmol IMP or TIMP/mmol Hb/hr; V_{max}/K_m: 1000/mmol Hb/hr

The results showed that both ITP and TITP are substrates for ITPase. Pyrophosphohydrolysis of ITP and TITP followed Michaelis-Menten kinetics: this is shown in Figure 6.3 for the normal (wild-type) genotype. The other polymorphisms showed similar kinetic behaviour.

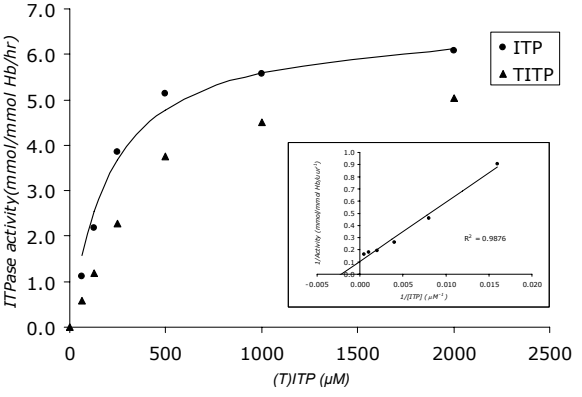

Figure 6.3 Michaelis-Menten kinetics of ITP and TITP in the normal *ITPA* genotype; insert Lineweaver Burk plot for ITP.

With the exception of the compound heterozygous c.94C>A/g.IVS2+21A>C polymorphism where the binding was less efficient, the substrate binding for ITP was identical in the different genotypes investigated. The velocity of pyrophospho-hydrolysis appeared to be genotype dependent: the wild type having the highest V_{max}, resulting in the most efficient generation of IMP. The heterozygous g.IVS2+21A>C genotype showed a slightly lower efficiency in the hydrolysis of ITP, the efficiency further decreasing when the second allele is also polymorphic for the g.IVC2+21A>C mutation. The results of this experiment also showed the deleterious effect of the c.94C>A mutation on the capacity of the protein to hydrolyse ITP. The protein resulting from the heterozygous c.94C>A genotype hydrolyzes ITP about 5 times less efficient than the wild type protein. Combined with the g.IVS2+21A>C allele the efficiency decreased to 5% of the wild type.

Our results for TITP showed that pyrophosphohydrolysis is as efficient as it is for the natural substrate ITP. To confirm this finding we measured human erythrocyte ITPase activity in the different genotypes using ITP and TITP as substrates. As can be seen in Table 6.3 the activities for ITP and TITP were comparable.

Table 6.3 Correlation between erythrocyte ITPase activity towards ITP and TITP in *ITPA* genotypes.

Substrate	ITP	TITP	
ITPA genotypes	Activity*	Activity**	ratio
c.94 CC /g.IVS2+21 AA	2.94	2.90	0.99
c.94 CA / g.IVS2+21 AA	0.62	0.69	1.11
c.94 CC /g.IVS2+21 AC	1.99	2.03	1.02
c.94 CA / g.IVS2+21 AC	0.20	0.33	0.63
c.94 CC /g.IVS2+21 CC	0.94	1.28	0.73

* mmol IMP/mmol Hb/hr;** mmol TIMP/mmol Hb/hr

Discussion

The *ITPA* gene is a polymorphic gene, several polymorphisms are described in the literature, some influencing the ITPase activity and others having little or no effect. Since *ITPA* polymorphisms are associated with thiopurine ADR, it is important to obtain more insight on the effect of *ITPA* gene polymorphisms on the kinetic behaviour of ITPase, towards both ITP and TITP. We therefore established genotype defined reference values for ITPase in our population (Table 6.1). As is shown in this table, our reference values are in line with earlier published values. A selection of these samples were also used for the mechanistic experiments. To the best of our knowledge, this is the first report on kinetic data for ITPase proteins originating from a variety of genotypes directly obtained from human materials for both ITP and the alternative substrate TITP.
As is shown in Table 6.1 the patients carrying the c.94C>A polymorphism, either in the heterozygous or homozygous state, have significantly decreased erythrocyte ITPase activities. The deleterious effect of the c.94C>A polymorphism is clearly demonstrated by the difference of the activity of the homozygous g.IVS2+21 A>C and the compound heterozygous c.94 C>A / g.IVS2+21 A>C variants where the ITPase activity decreases from ~32% to 8% of the wild type activity (Table 6.1). In addition we reported two, so far unknown, polymorphisms in the *ITPA* gene. One polymorphism, c.97T>C, shows the same influence on the ITPase activity as the c.94C>A mutation. It can be speculated that this alteration in the cDNA has the same effect on alternative splicing or affects the catalytic site in a similar mode as the c.94C>A mutation[18]. The other mutation, c.122A>G, did not alter erythrocyte ITPase activity.

From the kinetic data it can be concluded that the binding of the substrate is not altered, as in all investigated polymorphisms the K_m was comparable. The exception was the compound heterozygous c.94C>A/g.IVS2+21A>C genotype, which showed a significant lower affinity for the natural substrate. From our results we conclude that the mutated protein still possesses the normal binding capacity, as was predicted from the study on the ITP crystal structure by Stenmark et al.[15]. They also speculated that most probably the catalytic site of the P32T protein, originating from the c.94C>A polymorphism, is altered. Our data on the catalysis rate support this assumption. The V_{max} is significantly lower when the c.94C>A polymorphism is present alone or in combination with another activity lowering polymorphism. Surprisingly, with respect to the g.IVS2+21A>C variant the efficiency of the enzyme is decreased only in the homozygous state, the heterozygous variant shows the same kinetic properties as the wild type enzyme. This implies that this polymorphism only has a minor influence on the protein activity, only when both monomers originate from a g.IVS2+21A>C polymorphism the properties of the protein are altered.
In contrast to the results reported by Stepchenkova, we and Shipkova et al. found no substrate inhibition of ITPase by ITP in the wild type genotype[14,19]. We suppose that

the substrate inhibition in their experiments is possibly caused by the presence of DTT in the reaction solution or another artefact, influencing the pyrophosphate assay, rather than by true substrate inhibition[14]. DTT does not interfere in the HPLC-based methods measuring the formation of IMP, as is the case in the methods quantifying pyrophosphate generated in the assay.

It was proposed that the diminished activity of ITPase in the c.94C>A genetic variant is due to lowered levels of the protein[13,14]. Arenas et al. investigated the occurrence of alternative splice variants and reported less full length mRNA , resulting most probably in diminished amounts of the wild type protein[18]. When comparing our results on the c.94C>A variant with previous published data we propose that the protein, resulting from this polymorphism, has less pyrophosphohydrolysis capacity due to changes in the structure of the protein, affecting the catalytic site in particular.

Our results demonstrate that the pyrophosphohydrolysis of TITP by human erythrocyte ITPase in the normal genotype is as efficient as that of ITP. As a consequence, the accumulation of TITP may reach the same levels as ITP in patients with ITPase deficiency, however this needs further studies. No elevated concentrations of ITP have been demonstrated in individuals heterozygous for *ITPA* polymorphisms. Therefore we assume that TITP will not accumulate in cells from these individuals during thiopurine therapy, although a dose-dependent effect cannot yet be excluded. The ADR described during thiopurine therapy in patients carrying *ITPA* polymorphisms in our opinion cannot solely be attributed to intracellular TITP accumulation and it is therefore likely determined by other variables as well. A secondary function/influence of ITPase cannot be excluded. It can be speculated that ITPase is essential in maintaining the intracellular balance of non-canonical nucleotides, as in proliferative disorders it is over-expressed[20]. We propose that the house keeping function of the gene may be compromised under metabolic stress. When thiopurines are given to these patients the residual activity may not be sufficient to cope with this stress and results in ADR.

In conclusion, we have shown that polymorphisms in the *ITPA* gene influence the activity of ITPase in human erythrocytes. The c.94C>A variant had the greatest negative effect on the activity, resulting in a null activity for the homozygous state of this polymorphism. Whether this null activity is due to the decreased availability of mature protein or a true diminished ITPase activity, because of altered binding capacity or changes in catalytic properties, has to be further elucidated. The handling of TITP by ITPase is as efficient as that of ITP, indicating that accumulation of TITP will likely only occur in patients with ITPase deficiency. We speculate that the ADR in thiopurine therapy associated with *ITPA* polymorphisms is due to epigenetic factors rather than to TITP accumulation related to the decreased ITPase activity.

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