Chapter 8

General discussion

Introduction

Since the introduction of thiopurine as an anti-inflammatory drug about 50 years ago, a substantial amount of knowledge has been generated with respect to its activation, metabolization and the way it exerts its action[1]. Polymorphisms in genes involved in thiopurine metabolism result in different responses to thiopurine therapy, ranging from non-responsiveness, disease progression, to an overreaction with clinical consequences[2-8]. The studies described in this thesis have been focused on two enzymes which are involved in the metabolism of thiopurines, thiopurine-S-methyltransferase (TPMT) and inosine triphosphatase (ITPase).

Functional aspects of thiopurine-S-methyltransferase

One of the defense mechanisms the organism has to reduce the toxicity of xenobiotic compounds is methylation. Therefore a whole class of enzymes, called methyltransferases, exists with a broad spectrum of specificities[9,10]. TPMT is one of the enzymes belonging to this class and its main purpose is the methylation of thiopurine metabolites. Given the fact that thiopurines are man made and not existing in nature, one might wonder how the evolution of the gene encoding for the enzyme TPMT has taken place. It might be that a compound which is rather similar to the thiopurines still exists[11]. Early reports on TPMT tested several aromatic thiol containing compounds as substrates for TPMT and found that TPMT was capable to transfer the methylgroup from S-adenosyl methionine (SAM) to these compounds[12]. Freshwater bacteria use a TPMT-like pathway to methylate selenium-containing compounds, which appears to be a specific selenium methyltransferase[13,14]. Taking into account these findings it can be speculated that TPMT is involved in methylation of endogenous aromatic thiol and/or selenium containing compounds[11]. The methylation of thionucleotides was shown in earlier studies. However, at the start of our study it was still unclear whether thiopurine nucleotides are methylated inside the cell or if thiopurines are first methylated by TPMT and subsequently phosphoribosylated by HGPRT. Studies were initiated by incubating recombinant human TPMT (rhTPMT) with different thiopurine substrates, including thioinosine monophosphate (TIMP), and tritium labeled S-adenosylmethionine (SAM) as methyl donor, showing transfer of the labeled methyl group to the substrates. From the results obtained we were able to conclude that methylation of TIMP is as efficient as the methylation of the thiopurine bases, the incorporation of tritium was the same for all compounds, both after 6 and 24 hours of incubation.

Incubation experiments of MOLT-3 cells with different thiopurine species showed that 6-MTIMP is present intracellularly after 24 hours of incubation with 6-MP but not with 6-MMP. In the case of 6-MMP this means that 6-MMP is not phosphoribosylated by HGPRT in MOLT-3 cells. Studies with labeled precursors confirmed the results obtained

from the studies with rhTPMT. From our results we conclude that the generally accepted pathway for activation and deactivation of thiopurines is valid. Future experiments with TPMT deficient cell lines and the use of specific inhibitors of activating or deactivating enzymes can provide more insight in the way fluxes of thiopurines are directed through the pathways. The results from earlier studies with the xanthine oxidase (XO) inhibitor allopurinol and the inosine monophosphate dehydrogenase (IMPDH) inhibitor mycophenolic acid are good examples of such studies[15,16].

Determination of thiopurine metabolites

Analytical methods for the measurement of thiopurine metabolites are important tools in therapeutic drug monitoring (TDM)[17-19]. The aim of TDM in thiopurine therapy is to monitor the intracellular concentrations of the toxic compounds thioguanine triphosphate (TGTP) and methylthioinosine monophosphate (MTIMP)[20]. Both compounds are responsible for the observed ADR in thiopurine treatment: an intracellular excess of thioguanine nucleotides results in severe myelosuppresion, whereas an increased concentration of MTIMP is associated with liver dysfunction and inhibition of purine *de novo* synthesis[21].

For a better understanding of thiopurine metabolism it is of crucial importance to further investigate the distribution of thiopurine metabolites with respect to activation, deactivation and degradation. In the last decade liquid chromatography combined with tandem mass spectrometry was introduced in clinical chemistry and pharmacology. This technique makes it possible to measure metabolites at low concentrations while requiring a minimum of sample pre-treatment[22]. Our goal was to develop and validate a method for the measurement of thiopurine degradation products in biological fluids. This approach allowed us to quantify 6-mercaptopurine (6-MP), 6-thiouric acid (6-TUA), 6-thioguanine (6-TG), 6-methylmercaptopurine (6-MMP) and 6-methylthioguanine (6-MTG) in urine and plasma. The applications of this method go beyond the measurement of these metabolites in body fluids, as it can be used in translational studies like intestinal absorption and transport of thiopurines in vitro. A lot of insight is still lacking on how thiopurines are handled by the intestine, especially during inflammation (M. Crohn and Colitis ulcerosa). We expect that thiopurine efficacy will decrease further during of inflammation, due to up-regulation of XO expression in the intestinal tract . Future studies with cultured monolayers of intestinal cells and biopsies mounted in Ussing chambers can provide more insight in intestinal thiopurine kinetics[23-26].

Pharmacogenetic significance of TPMT

Although the pharmacogenetic significance of TPMT in thiopurine therapy is widely accepted, it is still not common in clinical practice to test patients for a decreased TPMT activity or polymorphisms in the *TPMT* gene before installing thiopurine therapy[27-29]. The importance of pre-treatment testing is highlighted by the case described in chapter 4 and other cases[2,4,5,7]. We strongly advocate a more prominent role for pharmacogenetic testing in general, and TPMT and ITPase in particular, to prevent adverse drug reactions, determined by genetic polymorphisms[2,4,5,30-40]. Pre-treatment screening will not only prevent ADR and hence improve the quality of life of the patient to be treated, it has also important socio-economic implications. In a significant number of cases the patient is hospitalized for several days, requiring specialist care. The volume of cost reduction as a consequence of pre-treatment TMPT pheno- or genotyping is substantial[41-44]. Recently the European Commission published a report on the potential socio-economic impact of pharmacogenetics and pharmacogenomics in the European Union (EU)[45]. This report stressed several recommendations which need to be implemented in the near future. Only thereafter patients can successfully benefit from the advantages of pharmacogenetic testing. Education of the medical professionals on the subject is regarded as essential. Not all physicians are aware of the great advantages pharmacogenetics can provide in treating patients. So far in the UK only dermatologists have guidelines for TPMT screening in case of thiopurine treatment[27,29]. Currently TPMT screening is not common clinical practice in the Netherlands, only local initiatives are active in this respect[30]. Moreover, in order to successfully implement pharmacogenetic testing, the services of laboratories offering these test should be easily accessible. To achieve this it is necessary that the costs of pharmacogenetic testing are covered by health insurances, making it more attractive for physicians to use pharmacogenetic tests in patient care and management.

The role of ITPase in thiopurine metabolism

The role of inosine triphosphatase (ITPase) in human metabolism is still not fully understood. It is apparent that its primary role is the pyrophosphohydrolysis of ITP and dITP, in order to maintain the inosine nucleotide balance. In addition, ITPase is involved in the removal of non-canonical nucleotides, hereby maintaining RNA and DNA integrity[32,46].

It is this last property of ITPase which makes it an intriguing part of human metabolism. Complete deficiency of ITPase is not associated with a clinical condition in humans. Excessive intracellular concentrations of ITP are the only abnormality reported in ITPase deficient individuals[32,46-48]. However the house keeping function of ITPase, the removal of the non-canonical nucleotides, is thought to be associated with

the outcome in (auto)inflammatory disorders or, in the case of viral infections, with the absence of adverse drug reactions under anti-viral therapy[49,50]. In a recent study *ITPA* gene knockout mice were not viable, they died within two weeks after birth. Besides growth retardation, the main clinical feature was cardiac myofiber disarray, most probably due to ATP displacement by ITP in the ATP pool, indispensable for proper sarcomere function[51]. Although the murine *ITPA* knock-out model, in which the whole gene was deleted, is not comparable to the human *ITPA* polymorphism with a mutated protein, the finding of this report suggests an yet unknown structural function of ITPase.

In chapter 6 we focused on one aspect of the house keeping function of ITPase, e.g. the handling of thioinosine triphosphate (TITP). This thiopurine metabolite is formed from thioinosine monophosphate (TIMP) and accumulates inside the cell when ITPase is deficient. Whether it will accumulate in individuals bearing activity lowering polymorphisms is still unclear. In the literature conflicting results on the occurrence of adverse drug reactions due to thiopurine therapy have been reported[52,53]. We studied the handling of ITP and TITP by ITPase in individuals with the different *ITPA* polymorphisms to gain more information on the influence of these polymorphisms on ITPase activity. Surprisingly the binding of the substrate did not differ greatly for the different genotypes. As was shown in our study, the apparent K_m for ITP and TITP for the genotypes tested was comparable. However, the pyrophosphohydrolysis of ITP and TITP was clearly diminished when one of the alleles contained the c.94 C>A polymorphism. The c.94C>A polymorphism results in an amino acid change in the ITPase protein at position 32, a proline is substituted by a threonine. This results in a structural change of the protein which affects most probably the catalytic site[54]. A decrease in catalytic velocity may imply that the catalytic site remains occupied, which can result in higher intracellular concentrations of ITP or other non-canonical nucleotides. In line with this, it can be argued that this may compromise the housekeeping function of ITPase, as defined by removing the intra-cellular non-canonical nucleotides. Non-canonical nucleotides will be incorporated in RNA and DNA and lead to aberrations[55]. The integrity of RNA and DNA is essential for proper cell functioning and proliferation[56]. The importance of a balanced nucleotide pool is emphasized by Arczewska and Kusmierek in their review on the role of the bacterial *mutT* gene and its human orthologue hMTH1 in the proper functioning of DNA repair[57]. *MutT* negative bacteria were susceptible to higher mutation rates due to increased concentrations of 8-oxo-deoxyguanosine triphosphate, emphasizing the universal importance of a balanced nucleotide pool[56]. One line of research would be to study the effect of *ITPA* polymorphisms on intracellular nucleotide pools and chromosomal stability during development, both under physiological an non-physiological conditions.

Interestingly, the occurrence of an unexpected high number of interstitial deletions and intragenic rearrangements in the *DPYD* gene in patients with a deficiency of the enzyme Dihydropyrimidine dehydrogenase (DPD) has been described recently[58]. DPD is the rate-limiting enzyme in the pyrimidine degradation pathway. A deficiency of this enzyme causes accumulation of uracil and thymine. It can be speculated that an excess of uracil and thymine will have upstream effects in pyrimidine metabolism and will effect the intracellular pyrimidine nucleotide balance, disturbing proper cell proliferation and regulation. The presence of a fragile site, *FRA1E*, on chromosome 1 in the vicinity of the *DPYD* gene can be additive in the effect of the disturbed pyrimidine nucleotide balance in DPD deficient patients. Thymidine phosphorylase (TP), which is also a defect in pyrimidine metabolism, results in depletion of and multiple deletions in mitochondrial DNA (mtDNA). Excess thymidine will result in higher intramitochondrial concentrations of thymidine triphosphate (TTP) and subsequently a disbalance in the deoxynucleotide pool[59]. At present it is unknown if mechanisms analogous to the ones in DPD and TP deficiency are causative in the case of *ITPA* polymorphisms or ITPase deficiency.
The mechanisms described above affect DNA, either nuclear or mitochondrial. Arenas et al. reported an abnormal distribution of mRNA splice variants in *IPTA* polymorphisms, which was associated with specific splice enhancing or silencing sequences[60]. Future investigations may focus on ITPase protein expression associated with these abnormal splice variants.
Understanding of the above mentioned mechanisms is important with respect to the possible pharmacogenetic consequences of *ITPA* polymorphisms in thiopurine based therapy. In relation with thiopurine metabolism cellular experiments have to be repeated with precursors of TIMP, this can provide more insight in the role of thionucleotides in cell cycle regulation. The importance of *ITPA* polymorphisms in thiopurine therapy is described in a study by Stocco et al. where patients were treated with thiopurines for acute lymphoblastic leukemia (ALL) on basis of their TPMT genotype. The results of this study show that patients with an *ITPA* polymorphism had more ADR, e.g. severe febrile neutropenia, than patients with a *ITPA* wild type genotype[61]. The pharmacogenetic relevance of ITPase activity or *ITPA* polymorphisms was further strengthened by a recent report by Fellay et al. on the outcome of treatment in hepatitis C[49]. Patients with *ITPA* polymorphisms were protected against ribavirin induced anaemia. So far no in vitro studies on the role of *ITPA* polymorphisms in pharmacogenetics, and in thiopurine metabolism in particular, have been reported.

It is remarkable that most reports referring to ITPase activity or *ITPA* polymorphism are somehow related to (auto)inflammatory diseases[62-64]. This raises the question if ITPase or *ITPA* is involved in the immune response. In one study an overrepresentation of *ITPA* polymorphisms in a group of patients with inflammatory bowel disease (IBD) has been found[65]. We have observed an association between *ITPA* polymorphisms and pulmonary Langerhans' cell histiocystosis[66]. Compromised immunity is the key clinical

feature in two other disorders in purine metabolism: adenosine deaminase (ADA) deficiency which causes severe combined immunodeficiency (SCID) and purine nucleoside phosphorylase (PNP) deficiency which causes T cell immunodeficiency[67]. In both disorders there is intracellular accumulation of purine (deoxy)ribonucleotide triphosphates (dNTPs), analogous to ITPase deficiency. This excess dNTPs has consequences on various cellular processes. These include inhibition of ribonucleotide reductase, leading to inhibition of DNA replication and increased susceptibility for apoptosis due to activation of several aspartate-specific cysteine proteases (caspases) by dATP and dGTP[68,69]. The assumption that an accumulation of (d)ITP would not interfere in cellular processes is compelling, since dITP is demonstrated to be mutagenic[70].

How the immune system responds to an excess of intracellular (d)ITP can be studied in cell lines of patients with *ITPA* polymorphisms and control cell lines. Whether this response is different from the response seen in ADA and PNP deficient cell lines will be of special interest. In addition B- and T-cell populations of both wild type and polymorphic individuals need to be investigated to find more evidence for the hypothesis that ITPase and/or *ITPA* are involved in cellular immunity.

Conclusions

At the start of this thesis we formulated a number of questions. Results of the studies described in this thesis elucidated some of the questions and, if not conclusive, raised new questions which have to be addressed in the near future. It is apparent that thiopurine metabolism is far more complicated than suggested by the 'simple' metabolic pathway which is generally accepted. This resulted in the initiation of further research in this field, both mechanistic studies and patient related research. In addition there is the challenge to develop validated methods for the determination of thiopurine metabolites, both intra- and extracellular. Nothing is known on the role of other genes as inhibitors or effectors of *ITPA* so far, in depth molecular analysis of the human genome with new generation sequence techniques can reveal epigenetic factors. Expansion of the knowledge of thiopurine metabolism is the cornerstone for personalized drug regimens. The ultimate goal will be a more personalized medicine, resulting in better treatment, less adverse drug reactions and cost reduction in healthcare.

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