Chapter 1

Introduction

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Introduction

Purines and pyrimidines belong to a class of heterocyclic compounds, containing both carbon and nitrogen atoms in its ring structures. Purines are nine-atom heterocyclic molecules, pyrimidines contain six atom rings (Figure 1.1).

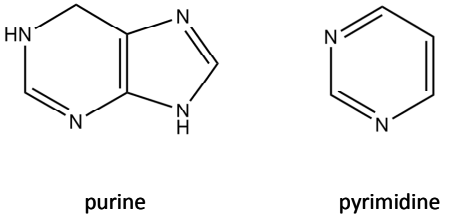

Figure 1.1 Basic chemical structures of purines and pyrimidines.

The nature of the atoms and side groups attached to the ring structures define the known purine and pyrimidine bases, nucleosides and nucleotides as is shown in Figure 1.2 for purines.

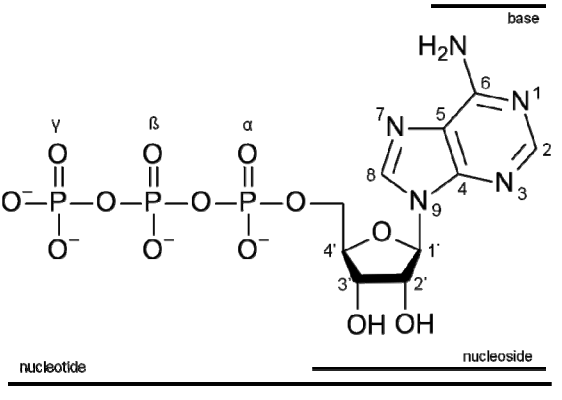

Figure 1.2 Molecular composition of purine base, nucleoside and nucleotide.

Purines and pyrimidines are of utmost importance for the maintenance of (human) life: purine and pyrimidine derivatives, nucleosides and nucleotides serve as building blocks for the nucleic acids RNA and DNA and play a major role in the cellular metabolism[1]. Table 1.1 shows the purine and pyrimidine constituents of DNA and RNA.

Table 1.1 Purine and pyrimidine derived nuclosides present in RNA and DNA.

Base	Nucleoside (RNA)	Deoxynucleoside (DNA)
Purine		
Adenine	Adenosine	Deoxyadenosine
Guanine	Guanosine	Deoxyguanosine
Cytosine	Cytidine	Deoxycytidine
Pyrimidine		
Uracil	Uridine	
Thymine		(deoxy)Thymidine

One of the most prominent cellular functions is the supply of high-energy phosphate esters (e.g. adenosine triphosphate, ATP) in phosphate transfer reactions, which are essential for the regulation of metabolism. Adenosine diphosphate (ADP) has a regulatory function in several enzymatic processes, e.g. oxidative phosphorylation[2]. Cyclic purine nucleotides, cyclic adenosine monophosphate (c-AMP) and cyclic guanosine monophosphate (c-GMP), are involved in signal transduction: an example hereof is the regulation of platelet aggregation by c-AMP[3]. Furthermore purine and pyrimidine mono-and diphosphate carbohydrates, like CMP-acetylneuraminic acid and UDP-galactose are required for the synthesis of macromolecules like glycoproteins and glycolipids[4]. Nucleotides are also part of several (co)enzymes like coenzyme A and flavin adenine dinucleotide (FAD).

It is clear that metabolism of purine and pyrimidine compounds needs to be tightly regulated to keep a balance between synthesis and demand of these compounds. Disturbances in the balance of these compounds will lead to disregulation of many cellular processes. Examples are inherited metabolic disorders of purine and pyrimidine metabolism and (acquired) derailed nucleotide metabolism in cancer cells[5].

The eminent role of purines and pyrimidines in cellular processes made them an interesting target for drug development, resulting in several classes of synthetic purine and pyrimidine analogues. These synthetic analogues are activated and metabolised along the same pathways as naturally occurring purines and pyrimidines and interfere with normal metabolism. This is the main reason that these compounds are used as therapeutics in a broad spectrum of diseases, in Table 1.2 an arbitrary selection of synthetic purine and pyrimidine analogues used as therapeutics is shown[6].

It is apparent that a genetic defect in one of the enzymes involved in the metabolism of purine or pyrimidine compounds may result in an altered response to purine or pyrimidine derived medication. The study of alterations in the metabolism of therapeutic drugs is known as pharmacogenetics. This topic will be discussed underneath in general and in more detail in the section on thiopurines and their metabolism.

Table 1.2 Selection of synthetic purine and pyrimidine analogues and their therapeutic application.

	Disease
Purine analogue	
	Inflammatory bowel disease
6-mercaptopurine, azathioprine,	Interstitial lung disease
6-thioguanine	Organ transplantation
	Leukemia
	Atopic dermatitis
Cladribine (2'-Chloro-2'-deoxyadenosine)	Haematological malignancies
Didanosine (2',3'-dideoxyinosine)	Acquired immune deficiency (HIV)
Abacavir	Acquired immune deficiency (HIV)
Pyrimidine analogue	
5-Fluorouracil	Colon and breast cancer
Gemcitabine (2',2'-difluorodeoxycytidine)	Lung and pancreatic cancer
	Solid tumours
Cytarabine (arabinosyl cytosine)	Haematological malignancies
Azidothymidine	Acquired immune deficiency (HIV)
Lamivudine (3TC)	Acquired immune deficiency (HIV)
Emtricitabine (FTC)	Acquired immune deficiency (HIV)

Purine and pyrimidine metabolism

Metabolism of purine and pyrimidines is an intricate network of biosynthesis, interconversion and degradation, which is tightly regulated by positive and negative feedback mechanisms. The aim of this regulation is to fulfill the demand of the organism for nucleotides, indispensable for replication and metabolic activity.

The central metabolite in purine metabolism is inosine-5'- monophosphate (IMP); this nucleotide is synthesised *de novo* in a sequence of 11 reactions, starting with the pyrophosphorylation of α-D-ribose-5-phosphate, catalysed by phosphoribosyl pyrophosphate synthase (PRPPS). The purine *de novo* synthesis (PDNS) reaction sequence is outlined in Figure 1.3.
PDNS is highly dependent on high energy phosphates: 4 reactions are ATP driven. In addition 2 reactions in this sequel are dependent on the folic acid cycle. The enzyme adenylosuccinate lyase (ADSL), responsible for the release of fumarate from aminoimidazole succinylcarboxamide ribonucleotide (AICAR), is a bifunctional enzyme and also active in the purine interconversion route where it is involved in the synthesis of adenosine-5'-monophosphate (AMP) from succinyl-AMP.
PNDS is controlled by feedback regulation of the first two steps by the products of the PDNS, AMP, guanosine-5'-monophosphate (GMP) and IMP. In addition pyrimidine nucleotides also inhibit PNDS, in order to maintain the balance between purine and pyrimidine nucleotides.

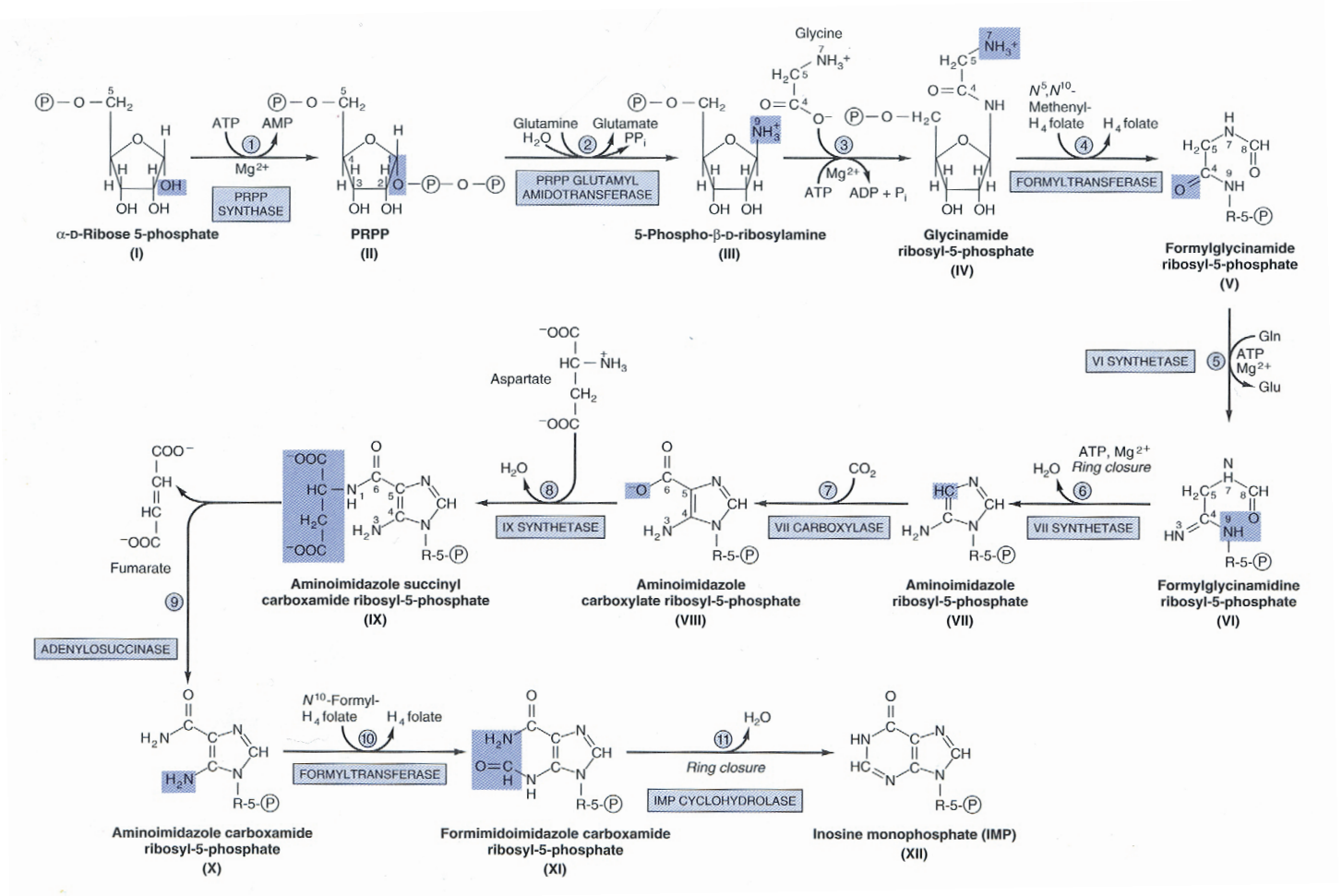

Figure 1.3 Purine *de novo* synthesis route.

Purine interconversion is the conversion of IMP, to either AMP or GMP (Figure 1.4). From these compounds the purine (deoxy)trinucleotides are synthesised, the building blocks for DNA and RNA, respectively.

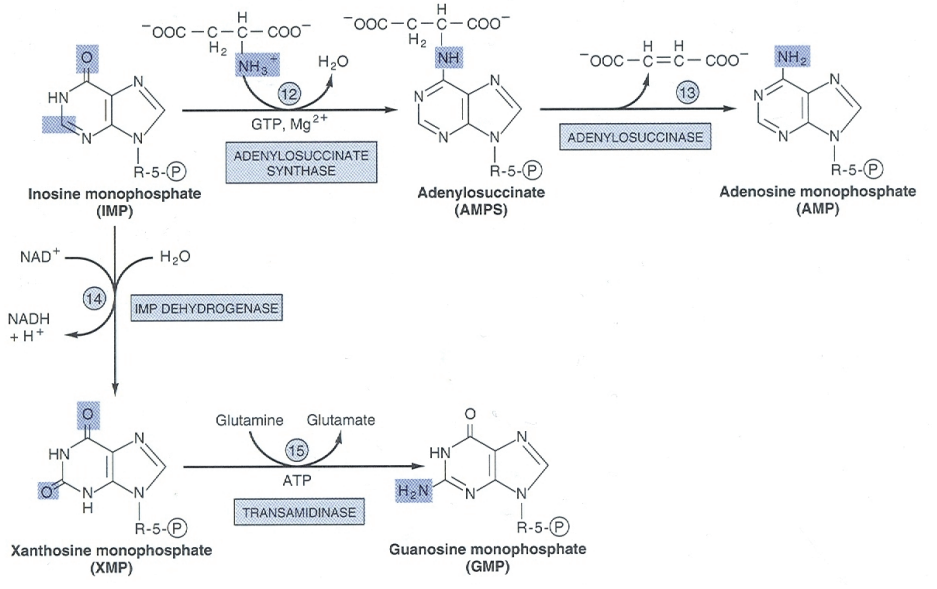

Figure 1.4 Purine interconversion route.

AMP is synthesised in two steps: IMP is converted to succinyl-AMP by adenylosuccinate synthase (ASS). Subsequently the succinyl moiety is spliced off by ADSL and AMP is generated. GMP is synthesised by conversion of IMP by inosine-monophosphate dehydrogenase (IMPDH) to xanthosine-5'-monophosphate (XMP), from which GMP is synthesised by guanosinemonophosphate synthase (GMPS). The majority of IMP is converted to AMP, which is reflected in the higher concentrations of the adenine nucleotides compared to the guanine nucleotides in cells. The balance between adenine and guanine nucleotides is achieved by feedback inhibition through AMP and GMP on the first enzymes in the interconversion route, AAS and IMPDH respectively. Furthermore ATP is essential for GMP synthesis and GTP is necessary for AMP synthesis, this so called 'reciprocal substrate relation' is a second regulator in the balance between adenine and guanine nucleotide synthesis.

The precise physiological role of ITPase in purine metabolism is unclear. In theory ITPase maintains homeostasis of IMP, the key compound in purine metabolism, by catalyzing the pyrophosphohydrolysis of ITP. However, no metabolic or clinical consequences of the accumulation of intracellular ITP are known. About 1% of the

general (western) population is deficient for ITPase, and because of the lack of a clinical phenotype it is assumed to be a benign condition[7-9].

A third mechanism to maintain purine nucleotide balance is the salvage pathway. As is shown in Figure 1.5 purines are degraded to hypoxanthine, guanine and adenine respectively by the action of different enzymes. These end-products can be recycled by hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and adenine phosphoribosyl transferase (APRT) into IMP, GMP and AMP respectively using phosphoribosyl pyrophosphate (PRPP) as the phosphoribosyl donor.

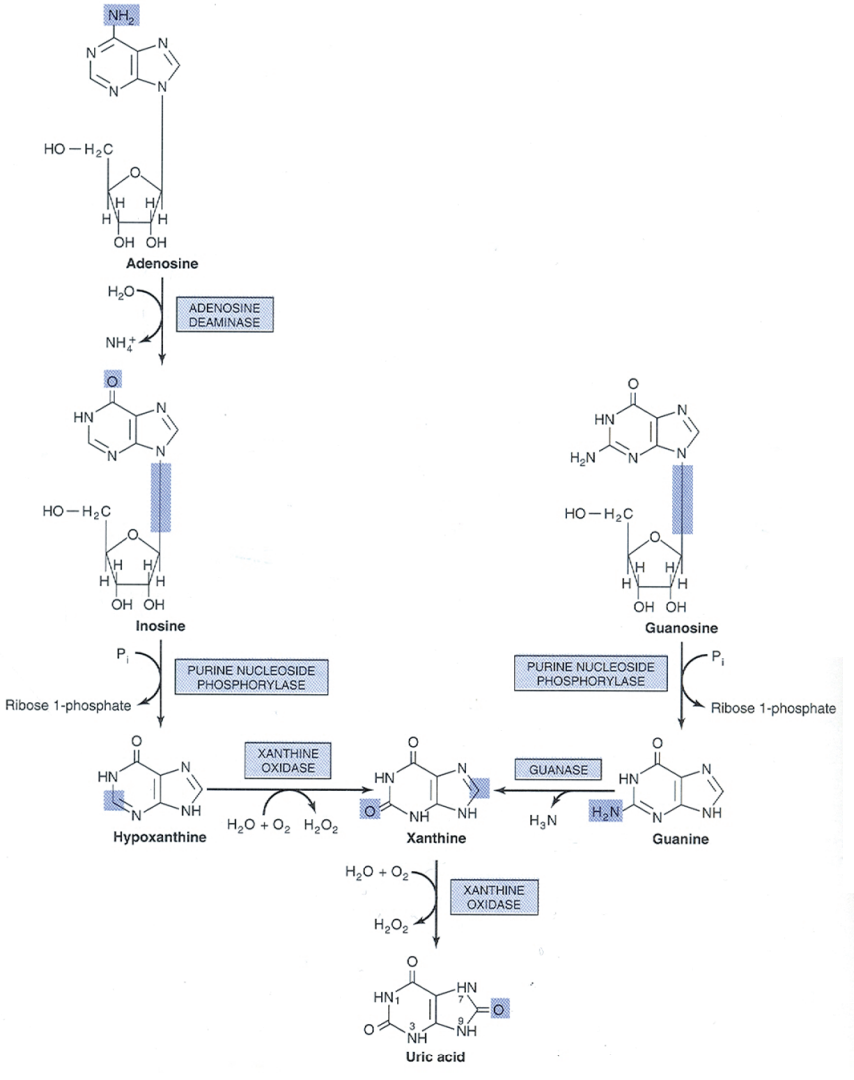

Figure 1.5 Purine degradation route.

The purine nucleotide pool in proliferating cells is maintained by the PNDS, in resting and non-nucleated cells the balance in the purine nucleotide pool is fully dependent on the salvage route.
Pyrimidine synthesis and metabolism differs greatly from purine nucleotide formation: first the pyrimidine ring is synthesised and subsequently attached to the ribose group. Because the focus is on purine metabolism, pyrimidine metabolism will not be further discussed. In the context of this thesis it is noteworthy to mention that the balance of purine and pyrimidine metabolism and synthesis is achieved by PRPP and purine nucleotides.

Pharmacogenetics

The way an organism, a person or an organ responds to medication depends on a complex of factors, including uptake, bioavailability, activation, deactivation, metabolism and clearance of the drug and its (deactivated) metabolites (Figure 1.6).

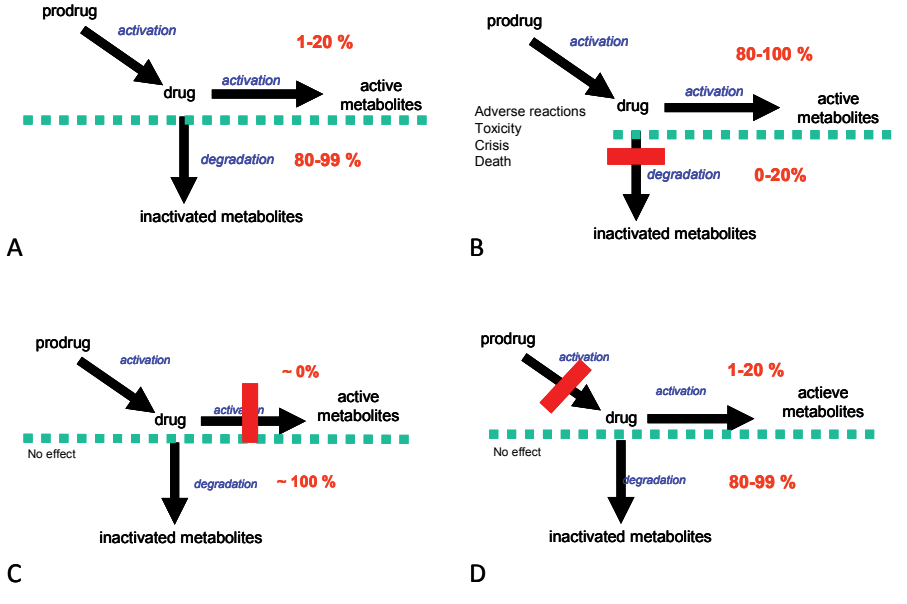

Figure 1.6 Interplay between activation, degradation (deactivation) and therapeutic effect of drugs: panel A: normal situation; panel B: impaired degradation, resulting in overreaction on therapy; panel C: defective activation, resulting in non responsiveness; panel D: impaired activation of prodrug, non responsiveness.

It is apparent that a disturbance of these factors will give an altered reaction to the medication, resulting in unwanted, uncomfortable or dangerous effects related to the use of the drug. These adverse drug reactions (ADR) are defined by the World Health Organisation (WHO) as: 'any noxious, unintended, and undesired effect of a drug that occurs in humans for prophylaxis, diagnosis, or therapy' [10]. Apart from non-genetic reasons, like (acquired) anatomical problems or un-controlled bacterial intestinal environment and drug-drug interactions, a significant number of ADRs is caused by genetic factors. Today inadequate response to a number of drugs is known to be caused by genetic polymorphisms. This concept is neatly outlined in a number of reviews[11-15]. As a result of the sequencing of the human genome, more information has become available about the way genes are organised and what their function is assumed to be. This new knowledge boosted pharmacogenetic research and resulted in a better understanding of the way drugs are handled by the human organism.
Besides monogenic traits with pharmacogenetic consequences, like dihydropyrimidine dehydrogenase deficiency (DPD), pharmacogenetics revealed the role of multiple genes and their corresponding proteins, in the metabolism of medication[15]. Although there are still many unanswered questions, several authors predict a prominent role for pharmacogenetics as the way towards personalised medicine. In their opinion, the response to medication can be predicted from the genetic make-up of a person[16-18].
Nowadays, the first steps in this regard are set: DPD pheno- and/or genotype of a patient is determined before the start of 5-Fluorouracil (5-FU) therapy in cancer to avoid fatal ADR[19]; *CYP* subclass genotyping is used to predict the response on often prescribed medications[12,20]. Pre-treatment screening for TPMT pheno- and genotype before commencing thiopurine is introduced as common clinical practice in several countries[21-23]. Although at present screening for TPMT 'only' accounts for 40% of the expected ADR in thiopurine therapy, the benefits for the patient and the health care system are already outreaching the costs for screening[24,25].
It may be imminent that in the near future more and more patients will be treated on guidance of their genetic makeup, thereby avoiding eventual life threatening ADR.

Thiopurines

Thiopurines are synthetic purines where the hydrogen atom at the 6 position in the ring is substituted by a sulphur group. Soon after their development in the 1950s by Gertrude Elion and George Hitchings, thiopurines found their application in clinical medicine. Three of these compounds are used as drugs for the treatment of a number of diseases : 6-mercaptopurine (6-MP, Purinethol®), the prodrug of 6-MP, azathioprine (AZA, Imuran®), and 6-thioguanine (6-TG, Lanvis®); the structures of these compounds are shown in Figure 1.7.

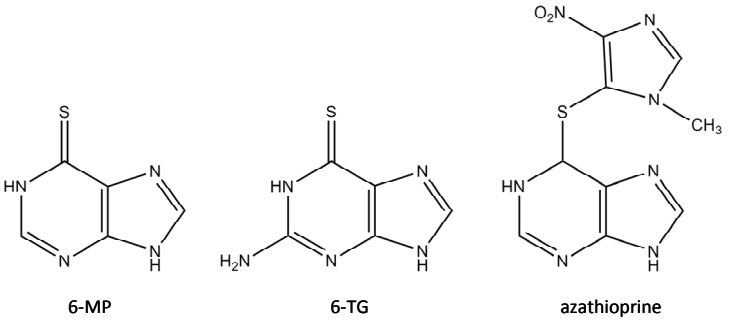

Figure 1.7 Commercially available thiopurine drugs.

AZA is widely used in the treatment of (auto)immune diseases, like inflammatory bowel disease (IBD), rheumatoid arthritis (RA), sarcoidosis, systemic lupus erythematosis (SLE), dermatological diseases and, before the introduction of more efficient drugs, in organ transplantation. 6-MP is routinely used in childhood acute lymphoblastic leukemia (ALL) and in case patients show allergic reactions due to the protective imidazole group in AZA. 6-TG is used in acute myeloid leukemia (AML) and ALL[6]. After activation 6-TG directly enters the guanine nucleotide pathway, therefore its immediate myelotoxicity is much greater than 6-MP and careful dosing of 6-TG is recommended.

Metabolism of thiopurines

The metabolism of thiopurines follows the same pathways as the natural occurring purines, with one addition: deactivation of thiopurines by thiopurine-S-methyltransferase (TPMT). In Figure 1.8 the current knowledge of thiopurine metabolism is outlined[26].

Not all enzymes are ubiquitously expressed throughout the organism, which means that the extent of thiopurine metabolism depends on the cell type. Erythrocytes exhibit TPMT, inosine triphosphatase (inosine triphosphate pyrophosphohydrolase, ITPase), adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP) and HGPRT activities, whereas nucleated cells also have purine-5'-nucleotidase (5'-NT), IMPDH, GMPS, xanthine oxidase (XO) and nucleotide kinase activities. The highest expression of XO is found in endothelial cells and liver. It is reported that systemic XO activity is highly dependent on external stimuli like metabolic stress[27].

As with natural purines, thiopurine handling depends on activation, deactivation, metabolism and intracellular thiopurine nucleotide equilibrium. To activate AZA, the prodrug of 6-MP, the imidazole group is removed, either by non-enzymatic degradation or, most probably, through glutathione-S-transferase (GST) activity.

A study with cultured HUVEC cells showed a decrease in glutathione concentrations after incubation with AZA, incubation with 6-MP did not alter the glutathione concentration[28].
The next step in thiopurine metabolism is the activation of 6-MP or 6-TG by HGPRT to the corresponding nucleotide monophosphates, thioinosine-5'-monophosphate (TIMP) and thioguanosine-5'-monophosphate (TGMP) respectively .
TGMP is further activated by mono- and diphosphate kinases and ribonucleotide reductase to thioguanosine-5'-triphosphate (TGTP) and thiodeoxyguanosine-5'-triphosphate (TdGTP) respectively, which are incorporated in RNA and DNA. TIMP needs further (inter)conversion through IMPDH and GMPS to generate TGMP.

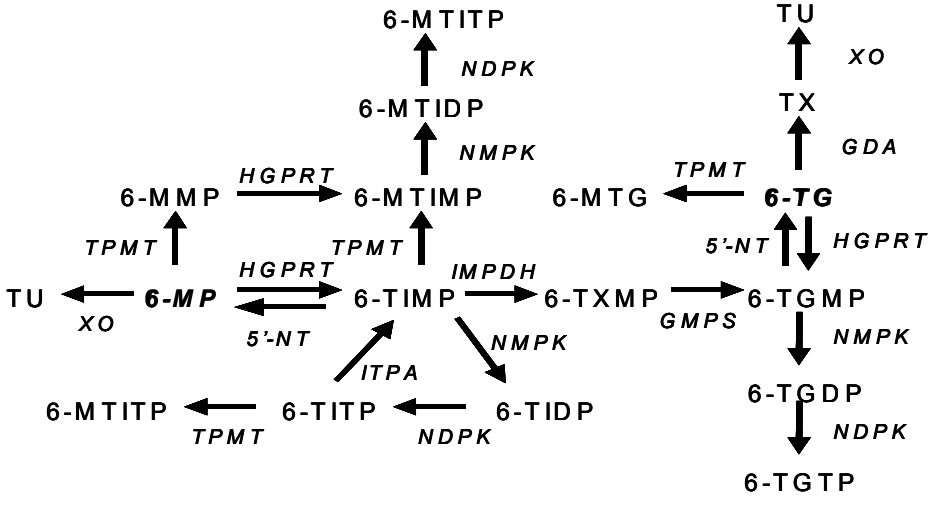

Figure 1.8 Generally accepted metabolism of thiopurines[26].

IMPDH is the rate-limiting enzyme of the interconversion of IMP to GMP, thereby regulating the necessary balance of adenine and guanine nucleotides inside the cell. The knowledge of the role of IMPDH in thiopurine metabolism is merely based on the inhibition of IMPDH by the immunosuppressant mycophenolic acid (MPA). This results in accumulation of TIMP, and, as is assumed, methyl-TIMP (MTIMP), a potent inhibitor of PDNS[29]. Recently a study was reported where a mutation in the promoter of *IMPDH1* resulted in resistance to thiopurine therapy, thereby illustrating the importance of IMPDH in thiopurine therapy[30].
The way GMPS plays a role in thiopurine metabolism is unknown. It can be predicted that a diminished activity of this enzyme will results in less efficacy of 6-MP and AZA.

If the response on 6-MP or AZA is less than expected, and the patient is committed to the therapy, 6-TG can be used instead. Although studies show that the way 6-MP and 6-TG exert their therapeutic action is somewhat different (see section on therapeutic action of thiopurines).

Deactivation is an important tool for an organism to control the concentrations of active and potentially toxic metabolites. In thiopurine metabolism several enzymes are involved in deactivation: 5'-NT, TPMT, PNP, XO and guanosine deaminase (GAD)[26].

Purine 5'-NT is active in nucleated cells like leucocytes, and therefore is an important regulator of thiopurine nucleotide concentrations in replicating cells[31]. Compared to pyrimidine 5'-NT the activity of purine 5'-NT in erythrocytes is negligible and will not be of importance for erythrocyte thiopurine metabolism.

Since the introduction of thiopurines in medicine, the role of the enzyme TPMT is the most extensively studied in thiopurine metabolism[32]. TPMT catalyses the transfer of a methyl group from a methyl donor (S-adenosyl methionine, SAM) to the sulphur atom of the thiopurine compound, 6-MP or 6-TG. Introduction of the methyl group at this position will deactivate the thiopurine molecule, most probably due to an alteration of the tautomeric configuration of the molecule. About 10-15% of thiopurines entering the circulation are deactivated by TPMT, so it is obvious that a deficiency, or even a lowered activity, of TPMT will have major consequences. The flux towards TGMP, catalysed by IMPDH and GMPS, will increase and results in enhanced intracellular concentrations of thioguanine nucleotides[33].

There is general agreement on the methylation of 6-MP and 6-TG by TPMT, but there is less consensus on the methylation of thiopurine nucleotides. In particular TIMP is thought to be methylated by TPMT because of the presence of MTIMP in extracts of cells that have been incubated with thiopurines. This was presented in earlier work on the characterisation of human TPMT[34,35], although later publications from other groups provided an alternative explanation for the presence of MTIMP. In an experiment published by Tay et al. MTIMP was detected in Ehrlich tumour cells after incubation with methylthioinosine (MMPR)[36,37]. This suggests that MTIMP originates from thioinosine (TI), rather than form TIMP. TI is formed through 5'-NT mediated phosphohydrolysis of TIMP. TPMT will methylate TI, which is phosphorylated by adenosine kinase (AK), resulting in MTIMP[28,38].

As is shown in Figure 1.8 PNP and XO are essential for the degradation of thiopurine nucleosides and bases to the metabolic end product 6-TU. It is estimated that degradation by XO accounts for over 80% of thiopurine metabolism of 6-MP administered. The role of polymorphisms XO was recently described by Hawwa et al., but unfortunately they performed no functional studies[39]. Most functional information of the role of XO until now is derived from reports on the use of allopurinol as co-medication with thiopurines. Allopurinol is a suicide inhibitor of XO, forcing all the 6-MP to be handled by TPMT and IMPDH. This will result in high intracellular concentrations of thioguanine nucleotides and methylated thiopurines[40].

The exact role of ITPase in thiopurine metabolism is still unclear, it is evident that thioinosine-5'-triphosphate (TITP) is hydrolyzed by ITPase[41]. Whether TITP accumulates in patients with partial ITPase deficiency is unknown. ITPase activity lowering polymorphisms in the *ITPA* gene are reported to be associated with ADR, although other reports cannot confirm these findings[39,42-45].
Although little is known on PNP involvement in thiopurine metabolism it is considered to be of importance. A lowered activity will result in high concentrations of thiopurine nucleosides and nucleotides, which are substrates for TPMT, resulting in abnormal concentrations of methylated compounds.
The function of guanine deaminase is thought to be minor, it is important when 6-TG is used as medication.

Therapeutic action of thiopurines

As is outlined above thiopurines need to be metabolised in several steps before they can exert their cytotoxic effect. 6-MP and 6-TG both are converted to thioguanine (deoxy)nucleotides (TGNs) and incorporated into RNA and DNA. Once incorporated into DNA these aberrant TGNs are responsible for the delayed cytotoxic action of thiopurines, causing DNA-protein interactions, due to the active sulphur group, interstrand cross-links and chromatide breaks[46].
The therapeutic efficacy of 6-MP is augmented further through its metabolite 6-MTIMP, this methylated nucleotide is a strong inhibitor of phosphoribosyl pyrophosphate amidotransferase, the first enzyme of the PNDS. This has several metabolic consequences. First there is depletion of the endogenous purine pool, e.g. ATP and GTP, having its effect on cellular processes depending on purine nucleotides. It will result in inhibition of cell growth: tumour cells treated with 6-MP were arrested in the late G_1 + S phase of the cell cycle[47]. A second effect, related to the PDNS inhibition, is the increased availability of phosphoribosyl pyrophosphate (PRPP). Increased PRPP concentrations will favour the conversion of 6-MP to 6-TIMP by HGPRT, and the subsequent toxic thionucleotides[48]. Furthermore the excess of PRPP will give an increase in pyrimidine synthesis, thereby altering the purine-pyrimidine nucleotide balance inside the cell. This will result in unbalanced growth and inhibition of cell growth.

Drug interactions in thiopurine metabolism

Like all drug metabolising pathways thiopurine metabolism can be altered by the products formed or competing drugs.
As already stated in the section on thiopurine metabolism the use of concomitant MPA or allopurinol will result in either the accumulation of TIMP and methylated analogues. In the case of allopurinol there will also be an aberrant increase in thioguanine nucleotides, resulting in myelotoxicity.
The use of combination therapies consisting of 6-MP or AZA and mesalazine (5-Aminosalicylate, 5-ASA) resulted in higher concentrations of TGNs after 4 weeks. Myelotoxicity rates were higher than in patients on thiopurine monotherapy[49]. TPMT is inhibited by 5-ASA and its acetylated metabolite, Ac-5-ASA. In addition there is information on inhibition of TPMT by sulfalazine, aspirin and other benzoic acid derivatives[6].

Objective of this thesis

Despite five decades of research there is still a lack in our knowledge of the metabolism of thiopurines and the way thiopurines exert their therapeutic efficacy. Considering the knowledge available we formulated a number of questions we wanted to be answered.
The first question, chapter 2, regarded the deactivation of thiopurines by TPMT, especially how 6-MTIMP is formed. This compound inhibits purine *de novo* synthesis at the level of the first step and therefore is an important metabolite in thiopurine metabolism. Is MTIMP generated directly by methylation of TIMP or are other mechanisms involved?
The measurement of thiopurine end metabolites is still cumbersome and only methods with limited analytical power are described. Therefore, our second goal was to establish and validate a method for the measurement of thiopurine metabolites using ultra performance liquid chromatography combined with tandem mass spectrometry and is described in chapter 3.
In chapter 4 the clinical consequences in a patient with a TPMT activity lowering polymorphism and thiopurine therapy are highlighted.
Measurement of the enzyme ITPase and its application in thiopurine therapy was the third objective of this study. More specifically the following questions were addressed in chapter 5: can dried blood spots be used as medium for the measurement of ITPase and if applicable, is the stability of the enzyme sufficient for retrospective measurement?

The influence of polymorphisms in *ITPA* are well described, on the other hand much less is known about the kinetic properties of the proteins originating from these polymorphisms. The handling of TITP by ITPase has not been described before, either for the wild type protein nor for the polymorphisms. The results of the experiments are described in chapter 6

There are some indications that ITPase, *ITPA*, or both are involved in the immune response. This assumption was studied in a group of patients with interstitial lung diseases and the results are described in chapter 7.

Acknowledgement: Figures 1.3-1.5 were used with permission from McGraw-Hill.

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