PART I

OPPORTUNITIES AND THREATS OF THIOPURINE METABOLISM AND DRUG MONITORING
CHAPTER 2

PHARMACOLOGICAL CONSIDERATIONS OF THIOPURINES IN THE TREATMENT OF INFLAMMATORY BOWEL DISEASE

This chapter is an updated compilation of two reviews:

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ABSTRACT

Thiopurines such as azathioprine, mercaptopurine and tioguanine are antimetabolites that have been used for several decades in the treatment of multiple diseases including inflammatory bowel diseases. Still many questions remain unanswered. Thiopurine metabolism is complex due to the involvement of multiple enzymes, of which the activities are genetically determined and cell type dependent. In addition, several methods of therapeutic drug monitoring have been developed in an attempt to relate drug availability of thiopurines with efficacy and intolerance. Some drug interactions may optimize metabolism of thiopurines and consequently increase its efficacy and decrease drug intolerance. This review focuses on pharmacology and drug monitoring of thiopurines and provides an overview of clinically and scientifically challenging topics concerning thiopurine therapy in IBD treatment. Topics discussed herein also include the topics that were discussed at the first meeting of the Thiopurine Task Force Interest Group which was held during the 2009 United European Gastroenterology Week in London (GASTRO2009). Thiopurines remain central to IBD treatment, although future studies are required to substantiate a more personalized medicine approach to their use.
INTRODUCTION

Thiopurines are widely used immunomodulating agents that were originally designed in the 1950’s for the treatment of childhood leukemia. Nowadays, these purine antagonists are used in the treatment of various diseases including rheumatoid arthritis, systemic lupus erythematoses and inflammatory bowel diseases (IBD). Both azathioprine (AZA) and mercaptopurine (MP) are effective in inducing and maintaining remission in Crohn’s disease (CD) and ulcerative colitis (UC). In addition, they reduce the need for corticosteroids and may postpone or even avert surgical interventions. Unfortunately, up to 40% of the patients fails thiopurine therapy within two years due to adverse events or resistance. Tioguanine (TG) is an effective alternative in AZA and MP intolerant or resistant patients but might be associated with nodular regenerative hyperplasia (NRH). Knowledge of the individual thiopurine metabolism, with determination of parameters indicative of clinical efficacy and toxicity, may be important to optimize therapy and reduce treatment failure. This strategy is known as therapeutic drug monitoring (TDM). Present-day TDM in thiopurine therapy suffers from several limitations, which are due to the complexity of pharmacokinetics, pharmacodynamics and pharmacogenetics. In addition, methodological limitations in the pharmacological analysis of thiopurine metabolites thwart interpretation. As a result, convincing correlations between currently used TDM parameters and parameters related with clinical efficacy and toxicity are scarce. Several drugs interact with thiopurine metabolism, which could potentiate adverse events, but could also widen the therapeutic range of thiopurines. Two important reviews have been published that addressed some of these aspects. The current review more specifically focuses on thiopurine metabolism and TDM, and provides suggestions to optimize thiopurine therapy in IBD patients.

METHODS

A PubMed search was conducted using the following MeSH terms: “azathioprine”, “mercaptopurine”, “tioguanine”, “drug monitoring” and “drug interactions”. The European database Embase was also searched for supplementary articles. English-language reviews, original articles, editorials, letters, abstracts and practical guidelines published between 1960 and 2009, were considered and additional articles were found in their reference lists. In addition, subjects discussed during the first meeting of the Thiopurine Task Force on thiopurine therapy in IBD, which was held in conjunction with the United European Gastroenterology Week in London in November, 2009, are reported. Topics were chosen for their clinical relevance and scientific interest.

THIOPURINE METABOLISM

Thiopurine metabolism is complex due to the involvement of multiple enzymes. In addition to the pharmacologically active 6-thioguanine nucleotides (6-TGN), several other (toxic)
metabolites are generated during this metabolization process. In recent years the insight into this metabolism increased considerably.

Pharmacokinetics and bioavailability
Azathioprine, MP and TG are pro-drugs that bear no intrinsic activity. After oral administration, bioavailability of AZA and MP varies between 30%-80% and 5%-37%, respectively[^14]^[15]. Availability of TG after oral administration is comparable to that of MP[^16]^[18]. The variation in bioavailability has multiple causes. Firstly, concomitant food intake can decrease the absorption of the thiopurines and can degrade thiopurines already before absorption[^19]^[22]. Secondly, and more importantly, an inter-individually varying xanthine oxidase (XO) activity in the gut and liver shunts MP away from bioactivation, as depicted in figure 1. Azathioprine escapes first-pass metabolism in the gut more effectively as compared with MP due to the fact that AZA is not a substrate for XO. After absorption, AZA is converted to MP for 88% and S-methyl-4-nitro-5-thioimidazole for 12%[^22]. Formerly, this conversion was thought to be a non-enzymatic reaction. Recently, however, it has been shown that 90% of this conversion is mediated by glutathione S-transferases (GST)[^23]. For both pathways, (reduced) glutathione (GSH) is used as a co-substrate. The molecular weight of AZA consists approximately 50% of MP and, taken the 88% conversion to MP into account, a conversion factor of 2.08 in weight is frequently used when calculating equivalent oral dosages of AZA and MP for clinical purposes (e.g. 1 mg/kg of MP is equivalent to 2.08

![Figure 1. Hypothesized first-pass metabolism. Both azathioprine (AZA) and 6-mercaptopurine (6-MP) are liable to a first-pass metabolism. This first-pass metabolism is for the greater part responsible for the differences in drug bioavailability. Mainly in the gut epithelial cells and liver cells xanthine oxidase (XO) catabolizes 6-MP to 6-thiouric acid (6-TUA). Azathioprine is not a direct substrate for XO in the gut. However, after its conversion to 6-MP, AZA is still catabolized to 6-TUA by XO in the liver. With the conversion of AZA to 6-MP, reduced glutathione (GSH) is oxidized and used as a co-substrate by glutathione S-transferase (GST). Finally, 6-MP is transported into the peripheral blood cells, in which 6-MP is further metabolized to the therapeutically active 6-thioguanine nucleotides (6-TGN).](image-url)
mg/kg of AZA). This conversion factor, however, is not adjusted to the aforementioned inter-individually determined first-pass effect. Once passing the liver, MP and TG are both metabolized intracellular via a complex pathway (purine salvage pathway) to the pharmacologically active 6-thioguanine nucleotides (6-TGN), as schematically illustrated in figure 2. It is this complex metabolism, together with inconsistent bioavailability, that makes the therapeutic range of thiopurine therapy narrow and hard to predict.

Cell specific thiopurine metabolism

Metabolism of thiopurines is dependent on cell specific availability and activity of involved enzymes. As an example, XO is primarily located in the gut epithelium and liver cells, but absent in red blood cells (RBC) and leukocytes, such as lymphocytes and leukemic cells. This gives thiopurines an advantage in target cells for immunomodulation and antileukemic activity. Drug specific metabolism is further illustrated by the fact that only 40% of TG as compared to 96% of MP is methylated by TPMT. Accordingly, RBC 6-TGN concentrations are more than six times higher on administration of an equivalent dose of TG as compared with MP. These results may in part be explained by a difference in affinity for TPMT between MP and TG. More convincing is the fact that RBCs have low inosine monophosphate dehydrogenase (IMPDH) activity, which limits the formation of 6-TGN out of MP. Duley and Florin assume that, when using AZA or MP, 6-TGN are indirectly and inefficiently incorporated after being metabolized by other hepatic and non-hepatic tissues, which possess normal IMPDH activity. With the usage of TG, RBC 6-TGN concentrations are relatively high due to the fact that RBCs have the ability to directly metabolize TG to 6-TGN via hypoxanthine-guanine phosphoribosyl transferase (HGPRT), without intervention of IMPDH.

Pharmacodynamics

In recent years, additional immunomodulating properties of thiopurines have been discovered. Originally, the mechanism of action of thiopurines was assumed to rely solely on the incorporation of thiopurine bases into DNA and RNA. 6-Deoxythioguanine triphosphate (6-dTGTP) is known to be the principle thiopurine derived base that is incorporated into the DNA strand upon replication. Although new interpretations continue to arise, the exact mechanisms by which the incorporation of these false bases translates into immunosuppression are not entirely clarified. DNA strand breakage, inhibition of replication, increased susceptibility to oxidation, interference with DNA methylation, interference with protein and nucleic acids synthesis and failure of the DNA mismatch repair system have been mentioned in this respect. In the treatment of IBD, in which thiopurines are relatively low dosed, it is denied that the immunosuppressive property of thiopurines is mainly effectuated by DNA incorporation of 6-dTGTP. Inhibition of purine de novo synthesis (PDNS) seems to be an additional immunomodulating property of MP and AZA. In this regard, 6-methyl thioinosine monophosphate (6-MTIMP) has been shown to strongly inhibit PDNS, whereas 6-methyl thioguanine (6-MTG) does not. In 2003, investigators found an additional effect...
Figure 2. Proposed thiopurine metabolism. Azathioprine (AZA) is converted to 6-mercaptopurine (6-MP). This reaction is mediated by glutathione S-transferase (GST) using reduced glutathione as a co-substrate. Once passing the liver, 6-MP is further metabolized intracellular via the purine salvage pathway. First, 6-MP is in part withdrawn from bioactivation by thiopurine S-methyl transferase (TPMT) that mediates the methylation of 6-MP to 6-methylmercaptopurine (6-MMP). Second, xanthine oxidase (XO) can catabolize 6-MP to 6-thiouric acid (6-TUA). It is estimated that in the majority of people, approximately 10% molar equivalent of a normal AZA dose is excreted as urinary 6-TUA. The bioactivation of 6-MP is mediated by hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and yields 6-thioinosine monophosphate (6-TIMP). 6-Thioinosine monophosphate can be further metabolized in three different ways. Firstly, 6-TIMP is a substrate for TPMT, which results in the formation of potentially toxic 6-methylmercaptopurine ribonucleotides (6-MMPR). These 6-MMPR include the potent purine de novo synthesis (PDNS) inhibitor 6-methyl thioinosine monophosphate (6-MTIMP), 6-methyl thioinosine diphosphate (6-MTIDP) and 6-methyl thioinosine triphosphate (6-MTTTP). Secondly, 6-TIMP can be phosphorylated by kinases via 6-thioinosine diphosphate (6-TIDP) to 6-thioinosine triphosphate (6-TITP), which in turn can be converted back to 6-TIMP by inosine triphosphate pyrophosphohydrolase (ITPase). Thirdly, 6-TIMP can be metabolized by inosine monophosphate dehydrogenase (IMPDH) into 6-thioxanthosine monophosphate (6-TXMP). This conversion might be a rate limiting step in the formation of the 6-thioguanine nucleotides (6-TGN) as determined in erythrocytes. 6-Thioxanthosine monophosphate, then, is converted by guanosine monophosphate synthetase (GMPS) into 6-thioguanine monophosphate (6-TGMP), which in turn is phosphorylated by kinases to 6-thioguanine diphosphate (6-TGDP) and 6-thioguanine triphosphate (6-TGTP). Together these nucleotides form the 6-TGN, which can be incorporated into RNA. 6-Thioguanine diphosphate is a substrate for ribonucleotide reductase yielding 6-thioguanine deoxydiphosphate (6-dTGDP) and subsequently 6-thioguanine deoxytriphosphate (6-dTGTP), which can be incorporated into DNA (not shown). The similarity between MP and TG bioavailability after oral administration suggests that TG is liable to a comparable first-pass effect in the gut, although TG is not a primary substrate for XO. Thioguanine is metabolized by guanine deaminase (GD) and aldehyde oxidase (AO) to 6-TX and 8-hydroxy-6-thioguanine (8-OH-6-TG), respectively. Subsequently, 6-thioxanthine and 8-OH-6-TG are catabolized by XO to 6-TUA. Thioguanine is partly methylated by TPMT to 6-methylthioguanine (6-MTG). In addition, in contrast to AZA and MP, TG is directly converted by HGPRT into the pharmacologically active 6-TGN.
of 6-thioguanine triphosphate (6-TGTP), especially in low-dose therapy. In CD28-stimulated T-cells, 6-TGTP binds and inhibits Rac1, which is a small GTPase that plays an important role in the inhibition of T-cell apoptosis. Normally, after binding guanine triphosphate (GTP), Rac1-GTP blocks apoptosis by inducing transcription of target genes such as those encoding mitogen activated protein kinase kinase, nuclear factor kappa B (NF-κB) and bcl-xL. However, when 6-TGTP binds Rac1 instead of GTP, this transcription is inhibited, leading to mitochondrial-mediated T-cell apoptosis. Following Rac1-6-TGTP combination, reduced Vav-Rac1 interaction suppresses the conjugation of T cells with antigen presenting cells by inhibition of lamellopodia formation and reduces Th1 cytokine production. In addition to the above mentioned mechanisms of action, 6-TGN also down-regulate the expression of important pro-inflammatory cytokines including tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), tumor necrosis factor receptor super family member 7 (TNFRS7) and alpha4-integrin in stimulated T-cells as identified by genome-wide expression profiling. Recently, clinically relevant thiopurine concentrations were also shown to inhibit proliferation of activated T cells in vitro. Furthermore, AZA possesses an immunosuppressive effect in addition to that attributable to MP and TG, which is thought to be the result of an immunosuppressive property of the methylnitroimidazol moiety. Immunomodulating properties of thiopurines are numerous and knowledge is still growing. Understanding the mechanisms of action creates opportunities for targeted and thus, more adequate TDM, which subsequently may result in less therapeutic failure.

Delayed response of thiopurines

The assumption that thiopurines have a delayed onset of therapeutic efficacy is widespread. Only after more than four months of therapy a clinical benefit of AZA and MP occurred. The 6-TGN half-life of several days or more, was initially thought to explain this delayed response. Hence, it was hypothesized that a loading dose of AZA could decrease the time to response. However, no increased frequency of early remission was found in those patients who received an intravenously administered loading dose of AZA. Steady state 6-TGN concentrations were already achieved by week two; a finding which was unexpected. In addition to the long half-life of 6-TGN, other mechanisms must be involved in this delayed onset of action of thiopurines. Firstly, the reduced Vav-Rac1 interaction following Rac1-6-TGTP combination, results in a decreased availability of functional Rac1 over time during thiopurine therapy by accumulation of Rac1-6-TGDP and Rac1-GDP. Secondly, the antiproliferative effect of thiopurines occurs only after five days independent from the thiopurine dose as T cell differentiation and cytokine production is not effectively inhibited until apoptosis. Moreover, only after 20 weeks of MP treatment, a marked decrease of memory T cell responses to a specific repeatedly encountered antigen occurs, whereas no such decrease of response was detected at earlier time points or after a previous non-recurring administered antigen. Thus, the delayed onset of therapeutic effectiveness of thiopurines is hypothesized not to be due to the duration of reaching steady state 6-TGN concentrations, but to a delayed reduction in memory T cell response to a repeatedly encountered antigen.
PHARMACOGENETICS IN THERAPEUTIC DRUG MONITORING OF THIOPURINES

Knowledge of the activity of enzymes involved in the metabolization of thiopurines may be helpful in explaining or predicting therapeutic success and toxicity. Several single nucleotide polymorphisms (SNP) in genes encoding these enzymes are linked with altered enzyme activities and therapeutic outcome. In recent years, not only SNPs in the TPMT gene, but also in genes encoding other involved enzymes have been identified.

Thiopurine S-methyl transferase (TPMT)

The most extensively investigated gene involved in thiopurine metabolism, is that of TPMT, located on chromosome 6. At present, two wild type and 25 variant alleles have been identified\(^5\). These variant or mutant alleles are characterized by SNPs in the open reading frame (ORF) of the TPMT gene and correlate with reduced TPMT activity, hence decreased 6-methylmercaptopurine ribonucleotides (6-MMPR) and increased 6-TGN concentrations are generated upon the use of an exogenous thiopurine drug\(^5\). Consequently, a higher risk to develop myelosuppression occurs. Thiopurine S-methyltransferase polymorphism is inherited as an autosomal co-dominant trait and differs between ethnic groups\(^5\). TPMT*3A is the most prevalent mutant allele in Caucasians and causes the largest decrease in enzyme activity, whereas TPMT*3C is the predominant allele in African or Asian subjects\(^13,53-55\). TPMT*2 is the last of the three most common TPMT mutant alleles and has only been identified in Caucasians. In a predominantly Caucasian population, TPMT phenotype is considered to be distributed in a trimodal manner, with approximately 90% of subject with a normal/high activity (13.50 ± 1.86 U/ml RBC), 10% with intermediate activity (7.20 ± 1.08 U/ml RBC) and 0.3%-0.6% with very low or no detectable enzyme activity\(^5\). By contrast, the distribution of TPMT mutant alleles in a Southeast Asian population seems to be unimodal\(^57,53\). The normal/high, intermediate, and low TPMT activity corresponds with two wild type alleles, heterozygote with one mutant allele, and compound heterozygote or homozygote with two mutant alleles, respectively. Interestingly, one to two percent of the 90 percent Caucasians with a wild-type TPMT allele exhibits ultra-high TPMT activity. Patients with this TPMT phenotype do not respond to normal dose AZA and MP and preferentially shunt toward the production of methylated products (6-MMPR) instead of 6-TGN\(^5\). Trinucleotide repeat variants in the promoter of TPMT relate with this ultra-methylation activity\(^5\). In addition, a variable-number tandem repeats within the TPMT promoter modulate TPMT activity\(^6\). A strong correlation between TPMT genotype and phenotype is critical if using TPMT genotype screening for the prediction of its activity. Schaeffeler and colleagues reported a concordance rate between genotype and phenotype of 98.4%\(^5\). This high rate, however, is owed to the fact that in patients with low TPMT activity without a common TPMT variant allele (TPMT*2, TPMT*3A or TPMT*3C), the entire TPMT ORF was sequenced to identify new allelic variants. Data on the concordance rate between genotype and phenotype are contrasting and may not be that strong when assessing only the three most common allelic TPMT variants. In a retrospective study from the United Kingdom, six out of 17 patients
with intermediate TPMT activity (phenotype) did not carry a variant allele (TPMT*2, TPMT3A and TPMT3B), giving a sensitivity of 65% and a specificity of 100% for TPMT genotyping predicting phenotype. At present, not only TPMT genotype screening, but also TPMT phenotype determination does not always correctly predict TPMT activity. Firstly, TPMT-activity in RBCs is subordinate to the age of the cells. Young RBCs exhibit higher TPMT activity than the older ones. Secondly, if a patient recently received donor blood for the treatment of anemia, RBC TPMT activity is likely to reflect the activity of the donor. Accordingly, in these cases genotype determination is recommended.

All TPMT compound heterozygous and homozygous IBD patients treated with normal dose thiopurines appear to develop myelotoxicity. However, only up to one third of the IBD patients with myelodepression during thiopurine therapy exhibit TPMT mutant alleles, indicating a multi-factorial cause for myelosuppression. This finding hampers TPMT-screening in clinical practice. Moreover, reduced TPMT activity is also related with other adverse events, such as gastro-intestinal complaints and rash. Besides eliciting adverse events, reduced TPMT activity relates with higher response rates in childhood leukemia and kidney transplantation when using thiopurines. Although IBD patients with intermediate TPMT activity had appreciably higher 6-TGN concentrations compared to those patients with a normal TPMT activity, there was no difference in TPMT activity between those in clinical remission and those with active disease. Recently, however, others actually observed that TPMT activity was statistically significantly lower in IBD patients who responded to AZA as compared to those who did not. This observation was corroborated by others.

Over the years, the suitability of TPMT screening before commencing thiopurine therapy has extensively been discussed. Matter of discussion is whether close monitoring of the hematological parameters, without knowing TPMT genotype or phenotype in advance, is appropriate enough to avoid severe myelosuppression. A large controlled clinical trial performed in the Netherlands showed that TPMT genotyping of the three most common variant alleles and the subsequent adjustment of the initial thiopurine dose does not result in overall fewer episodes of leukocytopenia as compared to standard dosing with frequent blood count testing. However, those with an acknowledged TPMT variant allele and initial dose adjustment had fewer chance to develop leukocytopenia than patients with such variant that received a standard dose of thiopurines (relative risk 0.11; 95% confidence interval 0.01-.085). As TPMT screening does not replace further toxicity monitoring and the frequency of TPMT deficient individuals is as low as 0.5%, the cost-effectiveness of TPMT screening was studied in pharmaco-economic studies. Several studies revealed a reduced time to respond to therapy as well as neutral or even reduced treatment costs, in particular when TPMT phenotype was assessed. The reduced time to respond reflects the initial higher thiopurine dosage in patients without inhibited TPMT function.

Current guidelines from the European Crohn's and Colitis Organization (ECCO) do not recommend TPMT genotype screening prior to thiopurine therapy, whereas the American Gastroenterological Association (AGA) does. As rapid TPMT genotype analyses are still not widely available, frequent monitoring of hematological parameters appears to be a safe
Monitoring of safety parameters, however, remains mandatory even if TPMT screening is routinely performed.

**Xanthine oxidase (XO)**

Xanthine oxidase (XO) is a cytoplasmic enzyme that catalyzes the oxidation of endogenous purines. Hypoxanthine is oxidized to xanthine, and xanthine to uric acid. Well-known exogenous substrates for XO are thiopurines, allopurinol and methylxanthines. Differences in the activity of XO, which in part result from genetic polymorphism, have implications with regard to individual variation in the bioavailability of thiopurines. Xanthine oxidase is distributed in many tissues and the highest activities are found in the liver and small intestine. Xanthine oxidase activity in liver tissue was found to be 21% higher in males as compared with females. In addition, a subgroup of approximately 24 patients exhibited a decreased XO activity. Recently, indirect evidence for a role of XO was obtained by Wong and colleagues, who described a case of a 55-year-old woman with autoimmune hepatitis. Despite good compliance to AZA and later MP she did neither produce 6-TGN nor 6-MMPR. *TPMT* genotype screening revealed a wild type genotype (*1/*1). Three weeks after the addition of the XO inhibitor allopurinol (100mg/day) to high dose MP therapy (2.5mg/kg/day), thiopurine metabolites exceeded the upper limit of the normal range and measured 1163 pmol/8x10^8 RBC and 10015 pmol/8x10^8 RBC for 6-TGN and 6-MMPR, respectively. This case clearly demonstrates that, although indirectly, if both 6-TGN and 6-MMPR concentrations are low despite adequate dosing of AZA or 6MP, XO activity may be radically enhanced. Three new SNPs in the XO genes of 96 participants were recently identified and the XO activities of 21 variant alleles were compared with the wild type. Ten of these variant alleles exhibited significant different enzyme activities as compared to the wild-type. Two variant alleles related with a deficiency in XO activity, six related with low activity and two related with a high activity. The most frequently observed variant alleles, however, did not affect XO activity. The XO variant allele 837C>T was shown to preserve patients from adverse drug reactions during thiopurine therapy. Notably, not only endogenous XO influences thiopurine metabolism. Cow’s milk contains high concentrations of XO, which decreases thiopurine bioavailability when ingested concomitantly. Whereas TPMT has extensively been studied in thiopurine therapy, relatively little is known about variation in XO activity. Based on what is known so far, genetic screening of the XO gene cannot be recommended in thiopurine therapy.

**Hypoxanthine-guanine phosphoribosyl transferase (HGPRT)**

At present, data concerning HGPRT activity, its relation with genetics and the implications for thiopurine metabolism are lacking. Hypoxanthine-guanine phosphoribosyl transferase catalyzes the first step of MP as well as TG toward bio activation (Figure 2). A rare disorder, in which HGPRT activity is absent, named Lesch-Nyhan syndrome, is characterized by abnormal metabolic and neurological manifestations. In these patients, thiopurines are presumably not cytotoxic. Several SNPs of the *HGPRT1* gene, that is located on the long
arm of the X-chromosome, have been identified. Hypoxanthine-guanine phosphoribosyl transferase is widely distributed throughout the body, but the nervous system in particular has very high enzyme activity. Recently, a case-control study in a Caucasian population comprising 422 IBD patients and 245 healthy controls was performed to identify SNPs in the genes encoding not only for TPMT and ITPase, but also for HGPRT1. Of the four HGPRT allele variants initially searched for, only one (rs14682666) has ultimately been evaluated, because the other three variants had a major allele frequency higher than 99%. No difference in HGPRT1 polymorphism was observed between IBD patients with or without adverse drug reactions and controls. However, since HGPRT activity may be influenced by inflammatory reactions, this might affect activation of MP and TG in target cells. Moreover, thiopurine therapy increases the mutation frequency of the HGPRT gene, which could theoretically explain thiopurine resistance in a few patients. Genetic screening for HGPRT mutations, however, cannot be implemented in thiopurine therapy so far.

**Inosine triphosphate pyrophosphohydrolase (ITPase)**

As an altered TPMT activity could only explain a part of all adverse events during thiopurine therapy, researchers continued to seek other explanations. Accordingly, it was hypothesized that reduced ITPase activity, due to SNPs in the encoding gene, could relate with adverse event. Normally ITPase catalyzes the pyrophosphohydrolysis of endogenous inosine triphosphate (ITP) to inosine monophosphate (IMP). Inosine triphosphate physiologically serves as a depot form of IMP, since ITP cannot be incorporated into DNA or RNA. Similarly, MP can accumulate as 6-thioinosine triphosphate (6-TITP), which is an inactive form that can be degraded by ITPase (Figure 2). Upon administration of AZA or MP, a deficiency in ITPase activity results in the accumulation of 6-TITP and subsequently 6-MMPR by methylation of 6-TITP. At present, five SNPs have been identified in the ITPase gene, which is located on the short arm of chromosome 20. Two of those SNPs (94C→A and IVS2+21A→C) are related with a deficiency of ITPase activity. Sumi and colleagues examined the ITPase gene in 100 healthy Caucasians and identified 10 subjects heterozygote for 94C→A, 24 subjects heterozygote for IVS2+21A→C and two compound heterozygote for these mutations. Homozygotes for the 94C→A mutations exhibit no ITPase activity whereas compound heterozygotes, 94C→A heterozygotes, IVS2+21A→C homozygotes and IVS2+21A→C heterozygotes exhibit a ITPase activity of 10%, 22.5%, 60% and 60%, respectively, when compared to the normal control mean.

Several studies were performed to explore whether ITPase deficiency relates with adverse events during thiopurine therapy. Initially, a case-control study, comparing 73 thiopurine treated IBD patients complicated by side effects and 74 thiopurine treated IBD patients without side effects, could not reveal an association between the 94C→A mutation and the occurrence of side effects. In contrast, another similar case-control study did show an association between the 94C→A mutation and the occurrence of certain side effects including rash, flu-like symptoms and pancreatitis. Notably, in this study all three
homozygotes for the $94C \rightarrow A$ mutation experienced various side effects. A cross-sectional study comprising 262 IBD patients showed an increased frequency of the $94C \rightarrow A$ variant allele in the leukocytopenic population as compared to those without leukopenia (16.7% versus 5.4%)\textsuperscript{104}. In addition to these above mentioned studies, one study observed an association between flu-like symptoms and the $94C \rightarrow A$ mutation\textsuperscript{71}; another study found an association between high 6-TGN concentrations and the $IVS2+21A \rightarrow C$ mutation\textsuperscript{105}; one concluded that thiopurine non-responders more frequently carry the $94C \rightarrow A$ mutation\textsuperscript{69}; and three could not ascertain an association between ITPase variant alleles and side effects during thiopurine therapy\textsuperscript{106-108}. Recently, a meta-analysis including six studies, did not show a correlation between the $94C \rightarrow A$ mutation and the development of thiopurine toxicity\textsuperscript{109}. Therefore, to date, insufficient data support routine determination of ITPase polymorphisms in thiopurine treated patients.

**Inosine monophosphate dehydrogenase (IMPDH)**

In purine metabolism IMPDH catalyzes the rate limiting step in the synthesis of guanine nucleotides via either purine salvage or via de novo synthesis. Two distinct types of human IMPDH isoforms (type 1 and 2) have been identified, the expression of which can be determined by real-time reverse-transcription PCR\textsuperscript{110,111}. In addition, enzyme activity can be determined by means of chromatography. In stimulated T-lymphocytes and in proliferating tumor cells both IMPDH micro-RNA expression and enzyme activity is enhanced\textsuperscript{112,113}. Existing data on IMPDH activity in healthy individuals as well as in certain patient groups are scarce and IMPDH activity seems to be cell specific\textsuperscript{29,114}. Moreover, studies which evaluated IMPDH activities are difficult to compare due to methodological differences. Recently, Haglund and colleagues showed that IMPDH activity (determined in peripheral blood mononuclear cells) of thiopurine using IBD patients was similar to that of healthy individuals\textsuperscript{115}. A negative correlation between IMPDH activity and 6-methyl thioinosine monophosphate (6-MTIMP) concentrations was observed, whereas no correlation with RBC 6-TGN concentrations could be found. In addition, no variant allele in the IMPDH1 or 2 genes has been identified that is related with IMPDH activity. Thus, at present there is no place for IMPDH activity measurement in clinical practice.

**NUCLEOTIDES IN THIOPURINE THERAPEUTIC DRUG MONITORING**

**Methodology**

Since the discovery of thiopurines, numerous high performance liquid chromatography (HPLC) procedures for the detection of thiopurine metabolites have been described. The method developed by Lennard in 1987 is the most extensively used method in clinical studies for establishing therapy related reference values of the thiopurine metabolites 6-TGN and 6-MMPR in RBCs\textsuperscript{116}. Five years later, this method was modified to allow for the simultaneous determination of 6-TGN and 6-MMPR in a single sample\textsuperscript{117}. As these methods are relatively
laborious, other researchers have sought to develop assays that can determine multiple metabolites in one run, including those that can determine individual 6-thioguanine phosphates\textsuperscript{118,119}. A more rapid and relatively easy to perform HPLC method for the determination of both RBC 6-TGN and 6-MMPR in a single run, is the method described by Dervieux and Boulieu\textsuperscript{119,120}. Dervieux’ method results in a more complete conversion of 6-TGN to TG. Shipkova and colleagues found a strong correlation when both HPLC methods were compared\textsuperscript{121}. However, Dervieux’ method led to a 2.6-fold higher 6-TGN concentration in RBCs. These findings suggest that putative therapeutic reference values are method specific. One must take into account that with the methods of both Lennard and Dervieux, the determined RBC 6-TGN concentration could originate from TG, 6-thioguanosine and the 6-thioguanine nucleotides, including the deoxynucleotides. Alternatively, the determined RBC 6-MMPR concentration is the sum of 6-methylmercaptopurine, 6-methylmercaptopurine riboside, 6-MMPR and the deoxynucleotides\textsuperscript{122}. Concentrations are usually expressed per number of cells, for instance per 8x10\textsuperscript{8} RBCs.

Limitations of these methods need to be taken into consideration. The stability of the nucleotides, when not frozen, is limited and temperature dependent. 6-Thioguanine nucleotide concentrations decreased 47% at day seven after storage at room temperature (22°C), whereas these concentrations decreased only 10% when stored at 4°C. The same holds true for 6-MMPR concentrations\textsuperscript{123}. Shipping conditions, thus, considerably influence metabolite concentrations. These variable shipping conditions together with different methods for determining metabolite values appear a common, but scarcely recognized flaw in the reproducibility and interpretation of metabolite concentrations in both clinical practice and research. Hence, it is not very surprising that studies could not ascertain a relation between clinical efficacy and 6-TGN concentrations\textsuperscript{124,125}. Moreover, in these studies metabolite concentrations are determined in a heterogeneous IBD population, characterized by variable courses of disease and variable thiopurine metabolizing properties.

Lennard and Dervieux initially perforce used RBCs to measure thiopurine metabolites in, as these cells remained available during high dose chemotherapy in leukemia patients. With the advent of other indications for thiopurines, methods were developed to determine metabolites in white blood cells (WBC)\textsuperscript{126-129}. This is of particular interest as these WBC, especially lymphocytes, are assumed to be the main target cells for thiopurine therapy. However, these methods are currently not feasible for routine clinical practice. Alternatively, RBC are easily obtainable and RBC 6-TGN concentrations appear to correlate with leukocyte 6-TGN concentration\textsuperscript{27,126}. Nevertheless, further developing methods of TDM using WBC is challenging and may be promising, the more so since WBC and RBCs have specific thiopurine metabolizing capacities and different cell volumes; properties which make comparability between these two cells difficult and unwanted (table 1). Although RBC 6-TGN concentrations differed markedly with equivalent dosages of MP and TG, WBC concentrations were nearly similar. Besides having a different metabolism, WBC have a much greater cell volume that RBCs. Accordingly, metabolite concentrations expressed per 8x10\textsuperscript{8} cells are expected to be greater than those found in the same number of RBCs.
Although RBC 6-TGN concentrations are related with therapeutic outcome to some extent, they are not the ideal parameter for TDM of thiopurine therapy. As more adequate alternatives are lacking, it is of considerable importance to optimize present-day TDM, for instance by improving operating procedures, such as cooling conditions and maximal storage time. When more sensitive and feasible methods are available, it will be of great interest to monitor thiopurine metabolite concentrations in WBC, the target cells, and relate their outcome with therapeutic efficacy and toxicity.

**Table 1.** Thiopurine metabolite concentrations in red blood cells (RBC) and white blood cells (WBC) during both MP and TG

<table>
<thead>
<tr>
<th></th>
<th>MP (75 mg/m2)</th>
<th>TG (43 mg/m2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>6-TGN (RBC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pmol/8x10^8 cells</td>
<td>261</td>
<td>1472</td>
</tr>
<tr>
<td>pmol/ml*</td>
<td>3625</td>
<td>20444</td>
</tr>
<tr>
<td>6-TGN (WBC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pmol/8x10^8 cells</td>
<td>5427</td>
<td>5142</td>
</tr>
<tr>
<td>pmol/ml*</td>
<td>7538</td>
<td>7124</td>
</tr>
<tr>
<td>6-MMPR (RBC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pmol/8x10^8 cells</td>
<td>7553</td>
<td>0</td>
</tr>
<tr>
<td>pmol/ml*</td>
<td>104903</td>
<td>0</td>
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<tr>
<td>6-MMPR (WBC)</td>
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</tr>
<tr>
<td>pmol/8x10^8 cells</td>
<td>3889</td>
<td>0</td>
</tr>
<tr>
<td>pmol/ml*</td>
<td>5401</td>
<td>0</td>
</tr>
</tbody>
</table>

* As RBC and WBC have different cell volumes, we calculated the metabolite concentrations per milliliter of cell volume assuming a RBC volume of 90 fl and a WBC being a spheroid and having a diameter of 12 µm. (adapted from Lancaster et al. 27)

Although RBC 6-TGN concentrations are related with therapeutic outcome to some extent, they are not the ideal parameter for TDM of thiopurine therapy. As more adequate alternatives are lacking, it is of considerable importance to optimize present-day TDM, for instance by improving operating procedures, such as cooling conditions and maximal storage time. When more sensitive and feasible methods are available, it will be of great interest to monitor thiopurine metabolite concentrations in WBC, the target cells, and relate their outcome with therapeutic efficacy and toxicity.

**TDM of azathioprine and mercaptopurine**

One of the first studies that aimed to correlate thiopurine metabolite concentrations with treatment outcome was reported by Cuffari and colleagues, in which an inverse correlation between 6-TGN concentration and disease activity was established in a cohort of 25 CD patients receiving MP128. Subsequently, 6-TGN concentrations above 235 and 250 pmol/8x10^8 RBC were associated with an increased frequency of therapeutic response in pediatric IBD patients130. and adult CD patients131, respectively. Comparable findings were reported by others, some of whom observed that a 6-MMP/6-TGN ratio below 11 predicted clinical response66,87,133-135. However, in several other studies a relation between metabolite concentrations and treatment response could not be obtained76,135-139. A meta-analysis comprising 12 studies eventually showed that patients with 6-TGN concentrations above the threshold values (230-260 pmol/8x10^8 RBC) were more likely to be in remission (62%) than those patients below the threshold value (36%) (pooled odds ratio 3.3, 95%CI 1.7-6.3, P<0.001)140. Unresponsiveness despite having 6-TGN concentrations above the threshold
value could be explained by different proportions of the separate thioguanine phosphates. Patients with relatively high concentrations of 6-TGTP more often showed clinical response as compared with those who with relatively higher 6-TGDP concentrations\textsuperscript{141}. Although sensitivity and specificity of 6-TGN and 6-MMPR concentrations for predicting clinical efficacy is somewhat poor, it is accepted that these concentrations can at least reveal therapeutic non-compliance.

Apart from being related with therapeutic efficacy, 6-TGN concentrations are also related with toxicity; in particular myelotoxicity. The incidence rate of AZA induced myelotoxicity is around 5\%\textsuperscript{142}. In a subpopulation of IBD patients with leukopenia during thiopurine therapy, the mean 6-TGN concentration was 490 pmol/8x10\textsuperscript{8} RBC\textsuperscript{131,143}. Furthermore, in a large cohort of 364 IBD patients, 6-TGN concentrations above 400 pmol/8x10\textsuperscript{8} RBC increased the frequency of myelotoxicity\textsuperscript{72}. Not only high 6-TGN concentrations, but also high 6-MMPR concentrations relate with myelotoxicity. Gilissen and colleagues described a case of a 23-year-old female with CD who developed pancytopenia one month after commencing MP therapy\textsuperscript{144}. 6-MMPR and 6-TGN concentrations were 57,000 and 126 pmol/8x10\textsuperscript{8} RBC, respectively. Ultra-methylation activity of TPMT presumably led to these high concentrations of 6-MMPR, including the strong PDNS inhibitor 6-MTIMP. Similarly, patients who developed myelotoxicity had higher 6-MTIMP concentrations as compared to those without adverse events. 6-Methyl thioinosine monophosphate concentrations above 11450 pmol/8x10\textsuperscript{8} RBC increased the risk of developing myelotoxicity considerably\textsuperscript{72,107}. Hepatotoxicity is another well-known adverse event of thiopurines, of which the pathophysiology is not clearly understood. One possible explanation might be a (relative) depletion of the hepatoprotective anti-oxidant reduced glutathione (GSH), following the conversion of AZA to MP. In addition to this conversion, XO mediated steps produce free oxygen radicals which will be scavenged by GSH, thereby decreasing reduced GSH availability. Among AZA and MP treated patients the incidence of abnormal liver tests, including hepatotoxicity defined as liver test abnormalities above two times the upper limit of the normal range, was 9.7\% per patient year\textsuperscript{145}. Although hepatotoxicity also occurs at low 6-MMPR concentrations, the risk increases above 6-MMPR concentrations of 5700 pmol/8x10\textsuperscript{8} RBC\textsuperscript{58,130,131}. This especially occurs in patients who, due to high TPMT activity, preferentially metabolize MP to the 6-MMPR. Upon dose escalation 6-TGN concentrations remain low in these patients, whereas 6-MMPR can extremely rise\textsuperscript{59,146}. Besides 6-MMPR, 6-TGN concentrations may also correlate with hepatotoxicity, in particular with nodular regenerative hyperplasia (NRH)\textsuperscript{147}. Nodular regenerative hyperplasia has been described not only with TG therapy but also with the use of AZA and even in a cohort of thiopurine naïve IBD patients\textsuperscript{6,148-155}. Breen and colleagues reported NRH in two liver transplant recipients who were on long-term AZA and both were heterozygous for a TPMT variant allele. Most likely 6-TGN concentrations were elevated, implying a causative role for 6-TGN in NRH development\textsuperscript{156}.

Both 6-TGN and 6-MMPR concentrations can be helpful in the explanation of therapeutic inefficacy, toxicity and non-compliance of AZA and MP. However, determination of these metabolites is currently not widely implemented in standard clinical practice.
TDM of Tioguanine

In the case of TG therapy, there is hardly any evidence on the possible relation between therapeutic outcome and thiopurine metabolite concentrations. Although RBC 6-TGN concentrations during TG therapy are several times higher than during AZA or MP therapy, leukocyte concentrations are almost similar. Accordingly, higher erythrocyte 6-TGN threshold values are likely to occur. Although TG has shown to be effective as a rescue drug, still a meaningful correlation between clinical efficacy and 6-TGN concentrations has not been obtained\(^\text{157-162}\).

In contrast to the literature concerning AZA and MP therapy, data on the development or incidence of myelotoxicity during TG therapy are scarce. Although TG therapy is associated with higher erythrocyte 6-TGN concentrations, the incidence of myelotoxicity is found to be equal to that found during AZA and MP therapy. It is therefore not surprising that 6-TGN concentrations above 450 pmol/8x10^8 RBC during TG therapy were not found to be indicative for myelotoxicity\(^\text{163}\). In addition, there seems to be no correlation between other adverse events and 6-TGN concentrations\(^\text{164}\). Hepatotoxicity, however, remains a major concern in the use of TG. Several studies are published that report a high prevalence of NRH during TG therapy in IBD patients\(^\text{10,11,165,166}\). The TG dose used in these studies was 40-80mg. Recently, others, including our group, did not report NRH in liver biopsies from IBD patients who had been using a median daily TG dose of approximately 20mg\(^\text{162,167-169}\). When dosing TG in daily doses of 20mg and 40mg, mean 6-TGN concentrations have found to be around 950 and 1650 pmol/8x10^8 RBC, respectively\(^\text{170}\). These findings suggest that the occurrence of NRH may be TG dosage or 6-TGN concentration dependent. Accordingly, with the use of TG, somewhat arbitrarily, we advocate an upper-limit of 1200 pmol/8x10^8 RBC.

**DRUG INTERACTIONS**

**Allopurinol**

The hypoxanthine analogue 4-hydroxypyrazolo (3,4-d)pyrimidine (allopurinol) is a potent XO inhibitor. Allopurinol itself is converted by aldehyde oxidase to its major metabolite oxypurinol, which is even a more potent XO inhibitor, that has a very long plasma half-life (18 to 30 hours)\(^\text{171,172}\). In order to improve MP therapy of childhood leukemia, allopurinol was combined with MP to inhibit degradation to 6-TUA and improve MP activation. Indeed, with the addition of 300mg allopurinol to 150mg MP only 3-4% of MP was excreted as 6-TUA with the urine, whereas without allopurinol 25% of MP was excreted as 6-TUA\(^\text{173}\). At the same time they found a 4-fold increase of urinary free MP with the addition of allopurinol, which reflects enhanced bioavailability. This enhanced bioavailability was also observed by other researchers\(^\text{174}\). An important question, however, is whether the addition of allopurinol increased therapeutic efficacy or just dose economy. Initially, the (chemo-) therapeutic index was thought to be increased as the addition of allopurinol seemed to potentiate the antitumor and immunosuppressive properties of MP three to fourfold, whereas toxicity was potentiated...
only twofold. Other investigations showed that the increased MP activity was accompanied
by a proportional increase in toxicity\(^1\). Thus, although less MP was required for comparable
efficacy, the (chemo-) therapeutic index remained unchanged. Consequently, the addition
of allopurinol to thiopurine therapy was abandoned. Since XO is also responsible for the
formation of uric acid from hypoxanthine and xanthine, allopurinol is widely used for the
treatment of gout and other forms of hyperuricemia. This wide use of allopurinol led to
unwanted combinations with thiopurines, without realizing the risk of developing dangerous
myelodepression\(^{175,176}\). Consequently, it is advised not to use this combination, at least not
without the reduction of the thiopurine dosage\(^{177-179}\). Although thiopurine dose reduction
reduces the risk of developing myelotoxicity during the use of allopurinol, this risk is not
abolished and still probably higher than with normal dosed thiopurine monotherapy\(^{180}\).

In 1993, Chocair and colleagues conducted a trial in which they compared “triple” therapy
(cyclosporine, prednisolone and AZA) with “triple” therapy combined with low dose
allopurinol in renal transplant recipients\(^{181}\). Interestingly, only one episode of rejection was
observed among the twelve allopurinol-treated patients with adjusted AZA dose, whereas
eleven of the fifteen controls had episodes with symptoms of graft rejection. In one other
study, again with renal transplant recipients (N=27) receiving AZA in combination with two
other immunosuppressive drugs, 6-TGN and 6-MMPR concentrations were determined\(^{182}\).
Six patients concomitantly received allopurinol in a dose of 100mg daily or every other
day. This study showed that in those patients with allopurinol, despite a dose reduction of
AZA (mean dose 33mg/day), 6-TGN concentrations were statistically significantly higher
compared to those patients without allopurinol and were equal to 363 and 122 pmol/8x10\(^8\)
RBC, respectively. No differences in 6-MMPR concentrations were detected. Recently,
Sparrow and colleagues described a cohort of fifteen IBD patients, who did not respond
to thiopurine therapy due to preferential thiopurine metabolism towards methylation\(^{183}\).
All patients received 100mg allopurinol daily and AZA/MP was reduced to 25-50% of the
original dose. After initiating allopurinol, 6-TGN concentrations predictably increased
from a mean of 186 to 385 pmol/8x10\(^8\) RBC (P<0.001). Remarkably, a decrease in 6-MMPR
concentration was observed (from 10380 to 1732 pmol/8x10\(^8\) RBC, P<0.001). These findings
suggest that not only XO, but also TPMT may be inhibited. As allopurinol itself is assumed
not to inhibit TPMT activity in vitro, the oxypurinol metabolite oxypurinol riboside
monophosphate, a 6-oxo analogue of 6-TIMP, has been proposed to exhibit the TPMT
inhibitory effect\(^{183,184}\). However, this still needs to be proven. Besides the increase in 6-TGN
concentration, the decrease in 6-MMPR concentration is a desirable side effect for those
patients who risk hepatotoxicity and myelotoxicity due to high 6-MMPR concentrations.
More recently, extended results were published by Sparrow and colleagues\(^{185}\). They observed
a decrease in disease activity with the addition of allopurinol. In addition, besides enabling
a reduction in corticosteroid dosage, allopurinol led to normalization of transaminase
concentrations. This hepatoprotective effect of allopurinol, when using thiopurines, was
also observed by others\(^{186,187}\). This beneficial effect of adding allopurinol to MP in patients
with preferential shunting towards 6-MMPR was subsequently demonstrated in children\(^{188}\).
As mentioned before, thiopurine related hepatotoxicity might result from oxidative stress. Allopurinol may decrease the production of free radicals both through the inhibition of XO and by functioning as a radical scavenger, and as such protect against hepatotoxicity\textsuperscript{189}. The interaction between allopurinol and AZA or MP, hence, on the one hand creates the ability to continue maintenance therapy in those patients who otherwise would be forced to stop therapy due to safety concerns. On the other hand, patients who were thiopurine unresponsive due to a disadvantageous metabolism keep the opportunity to benefit from thiopurine maintenance therapy. In order to elucidate the exact mechanism by which allopurinol interacts with thiopurine metabolism and to establish the true role of allopurinol co-therapy in the treatment of IBD, further research is warranted.

5-aminosalicylic acid and its pro-drugs

Sulphasalazine, balsalazide, olsalazine and mesalazine (oral delayed release or sustained release) contain the therapeutic 5-aminosalicylic acid (5-ASA) moiety and have effectively been used for the treatment of UC\textsuperscript{190}. The use of 5-ASA in CD patients is controversial\textsuperscript{82,191}. Although in vitro, 5-ASA shares many of the pharmacological properties of the non-steroidal anti-inflammatory drugs, the exact mechanism of action of 5-ASA has not completely been elucidated\textsuperscript{192}. Since benzoic acid inhibits TPMT\textsuperscript{193}, Szumlanski and Weinsilbom hypothesized that 5-ASA compounds, which are derivatives of benzoic acid, could also inhibit TPMT activity. They showed that in addition to 3,4-dimethoxy-5-hydroxybenzoic acid, sulphasalazine, 3-, 4- and 5-ASA inhibited TPMT activity by 50% (IC\textsubscript{50}) at 78, 99, 2600 and 1240 \textmu mol/L, respectively\textsuperscript{194}. The IC\textsubscript{50} to inhibit TPMT of olsalazine was as low as 23 \textmu mol/L and that of balsalazide was 197 \textmu mol/L\textsuperscript{195}. Notably, a CD patient developed myelodepression during MP therapy together with olsalazine\textsuperscript{196}. The clinical relevance of this TPMT inhibitory effect of these different 5-ASA compounds has been discussed by Green, who pointed out that with normal dosing of mesalazine, olsalazine and balsalazide, peak plasma concentrations of 5-ASA remained considerably below the reported IC\textsubscript{50} values\textsuperscript{197}. Alternatively, sulphasalazine (2.0g) dosed more than once daily is likely to reach peak plasma concentrations up to half the reported IC\textsubscript{50} values and should therefore be used with caution together with thiopurines\textsuperscript{198}. In vivo experiments showed that 6-TGN concentrations increased with concomitant usage of several 5-ASA preparations. Both the frequency of leukocytopenic events and 6-TGN concentrations increased markedly with the co-administration of both mesalazine and sulphasalazine but not balsalazide in CD patients using AZA or MP\textsuperscript{199}. Conversely, withdrawing the 5-ASA drug (sulphasalazine or mesalazine) in CD patients who had been using AZA reduced the median 6-TGN concentration, from 148 to 132 pmol/8x10\textsuperscript{8} RBC (P=0.027)\textsuperscript{200}; reintroducing 5-ASA to thiopurine therapy increased 6-TGN concentrations again\textsuperscript{201}. In 2007, De Boer and colleagues showed that 5-ASA interacted with thiopurine metabolism in a dose-dependent manner\textsuperscript{202}. Daily administration of 2g and 4g 5-ASA led to a statistical significant increase in 6-TGN concentration of 40% (absolute 84 pmol/8x10\textsuperscript{8} RBC) and 70% (absolute 154 pmol/8x10\textsuperscript{8} RBC), respectively. In accordance with other studies, 6-MMPR concentrations did not change in this study.
The enhancing effect of 5-ASA co-administration on 6-TGN concentrations is indisputable, although it is unclear whether this is only due to the inhibition of TPMT. Probably it is not, because in addition to increased 6-TGN concentrations, decreased 6-MMPR concentrations should also be ascertained. Nevertheless, the clinical relevance of this 6-TGN enhancing strategy is rather important. With rising 6-TGN concentrations, myelodepression is more likely to occur. However, the clinical benefit of the 5-ASA interaction is unclear, since in a retrospective study comprising 186 IBD patients relapse rates were similar between 103 patients using AZA with 5-ASA and 83 patients using only AZA. This study included both CD and UC patients. Another retrospective study showed that adverse events, AZA discontinuation rates and relapse rates were higher in those patients using 5-ASA concomitantly with AZA. These findings, which have recently been summarized in a systematic review, suggest that 5-ASA administration concomitantly with thiopurines has no clinical benefit. It must be noted, however, that within these retrospective studies confounding factors are very likely to have distorted the outcomes.

5-Aminosalicylates, thus, increase 6-TGN concentrations during thiopurine therapy, possibly due to inhibition of TPMT activity. This could influence the clinical outcome of thiopurine therapy in a beneficial as well as a disadvantageous manner, however well-conducted prospective trials are necessary to further address these questions.

Other combinations
Besides allopurinol and 5-ASA also several other drugs interact with thiopurines. Methotrexate (MTX) inhibits XO and thereby increases 6-TGN concentrations with thiopurine use. On a cellular level MTX may also enhance thiopurine activation by HGPRT. These interactions is potentially dangerous as it increases the risk of myelodepression without currently known clinical benefit. Infliximab increases 6-TGN concentrations within the first three weeks after the first infusion in thiopurine using IBD patients except for those using TG. The mechanism underlying this interaction is not clear and whether this particular phenomenon is clinically relevant is doubtful. Ribavirin, which is commonly used as a potentiator of peg interferon-alpha in the treatment of chronic hepatitis C, is a well-known inhibitor of the enzyme IMPDH. When administering ribavirin concomitantly with AZA or MP, an increased risk of developing myelotoxicity has been reported due to high 6-MMPR concentrations. Finally, diuretics, ACE inhibitors and NSAIDs possibly interact with thiopurines, albeit the clinical relevance remains to be determined.

ADVERSE EVENTS
The use of thiopurines has been associated with relevant adverse events (AE), some of which are dose dependent, whereas others are idiosyncratic reactions. Some of the dose dependent AE's may be prevented by TPMT screening, although thiopurine dose escalation may also avoid others. The most worrisome AE's are opportunistic infections, hepatotoxicity, malignancies, and pancreatitis, all of which may cause serious morbidity and mortality.
The most frequently observed opportunistic infections during thiopurine therapy in IBD patients are viral infections, such as herpes zoster, herpes simplex, cytomegalovirus and Epstein-Barr virus (EBV) infections\textsuperscript{213}. The predisposition to viral infections may be due to thiopurine-induced apoptosis of activated T lymphocytes, which are essential in the immunological defense against viruses. Patients using AZA/MP monotherapy have a three-fold risk of opportunistic infections as compared with controls. Combining thiopurines with corticosteroids increases this risk five-fold\textsuperscript{214}. Although absolute risks are low, a comprehensive vaccination strategy in IBD patients using thiopurines has been suggested, as outlined in a European consensus on the prevention, diagnosis and management of opportunistic infections in IBD\textsuperscript{215}. To assess more accurately the risk of infective complications in IBD patients using immunomodulators, it appears pertinent to study more fully the effects of these drugs on specific immunological functions, such as phagocytosis, lymphocyte functions and complement (de)activation \textit{in vivo}, as well as on vaccination protocols.

Hepatotoxicity events are well-known off-target effects of thiopurine therapy, which can be either a dose independent idiosyncratic reaction or a dose dependent reaction. As mentioned previously, high 6-MMRP concentrations have been associated with hepatotoxicity; the mechanism of which remains elusive. Endothelial cell injury may give rise to nodular regenerative hyperplasia (NRH) or veno-occlusive disease (VOD), both of which are associated with an increased portal pressure and its potential complications. A recent study in France showed a cumulative risk of clinically overt NRH in IBD patients using AZA of 0.5% at five years (95% CI, 0.11-0.89) and 1.25% at ten years (0.29-2.21). Male sex and a stricturing Crohn's disease behavior (resulting in small bowel resections) were identified as independent risk factors in multivariate analysis\textsuperscript{216}. However, this risk estimation was solely based on symptomatic cases of NRH and may not be different from the background age-adjusted prevalence. There is a relatively high background prevalence of NRH in non-thiopurine using IBD patients of 6%\textsuperscript{151}, and in a large post-mortem series (not including IBD patients) NRH was observed in 2.6%\textsuperscript{217}. Furthermore, little is known about the disease course of NRH, which may be indolent or progressive. Often (hepato-) splenomegaly and thrombocytopenia coincide with NRH. It is of clinical importance to know whether early detection of NRH provides a window of opportunity to prevent drug-related complications by withdrawing thiopurine therapy. In contrast, the identification of predictors of IBD course is of pivotal importance to prevent patients ceasing thiopurines unnecessarily. Endothelial cell injury is believed to have a central role in NRH etiology, but the detailed pathophysiological mechanism remains to be elucidated.

There is compelling evidence showing that IBD patients using thiopurines have an increased risk of developing a malignant Burkitt-like lymphoma, related to Epstein-Barr virus (EBV) infection. A meta-analysis of six studies, including 3891 IBD patients, showed a pooled relative risk of lymphoma during thiopurine therapy of 4.18 (95% CI, 2.07-7.51)\textsuperscript{218}. More recently, the CESAME study group from France published the results of their large prospective cohort study, comprising almost 20,000 IBD patients, suggesting that the use of thiopurines increased the calculated risk of lymphoma fivefold (HR 5.28; 95% CI, 2.01-13.9).
PHARMACOLOGICAL CONSIDERATIONS OF THIOPURINES IN THE TREATMENT OF INFLAMMATORY BOWEL DISEASE

The incidence ratios for patients of all ages who do and do not use thiopurines were 0.90 and 0.26 per 1000 patient years, respectively. For patients above the age of 65 years this ratio was 5.41. However, the same large prospective study demonstrated a 3.8-fold decreased risk of the more common colorectal cancer. There is emerging evidence that thiopurines should be co-prescribed with anti-TNF therapies, a combination that has been associated with rare cases of hepatosplenic T cell lymphoma predominantly in young male IBD patients.

However, hepatosplenic T cell lymphoma is not limited to exposure with thiopurine drugs with or without anti-TNF therapies. It is associated with immunocompromise generally and also occurs de novo. Explaining relative risks to patients is never easy and needs to be balanced with the need for treatment in properly selected patients. Overall, as compared with thiopurine monotherapy there seems to be no detectable additional risk signal of developing a malignant lymphoma when combining thiopurines with anti-TNF therapy. Whether the risk of lymphoma justifies a screening program can be debated. The absolute risk of lymphoma remains low and the overall clinical benefit of thiopurine therapy in IBD treatment seems to outweigh this risk, although the risk to benefit ratio is age dependent. As EBV may contribute to the development of malignant lymphoma during thiopurine therapy, it is of pivotal importance to understand better its role and to determine whether parameters such as viral load can predict the risk at developing a malignant lymphoma. In addition to the increased risk of lymphoma, thiopurine therapy has also been associated with non-melanoma skin cancers, which is why a strong sun protection program has been instituted in some countries at lower latitudes. Although different mechanisms might explain the carcinogenicity of thiopurines, more research is necessary to find ways to predict these serious complications.

Acute pancreatitis is a well-known and probably idiosyncratic adverse event of thiopurine therapy. The cumulative incidence of acute pancreatitis in IBD patients is around 1-2%, more than half of which can be attributed to the use of thiopurines. Thiopurine induced acute pancreatitis (TIAP) typically occurs within one month of treatment and is mild provided that the drug is ceased. In addition, female gender is a risk factor. At present, the pathogenesis of TIAP is not known, although it has been suggested that a deficient inosine triphosphate pyrophosphohydrolase (ITPA) activity may increase the risk of TIAP according to one study but not to others. Therefore, further investigation is required to create a better understanding of the mechanism behind this AE and provide knowledge to prevent its occurrence.

Tioguanine and nodular regenerative hyperplasia

In recent years, the use of TG has been advocated as a rescue drug for IBD patients failing to tolerate or respond to classical thiopurines. Proposed metabolic advantages compared to AZA and MP are the more direct conversion into 6-TGN, the limited influence of the enzyme TPMT and the absence of potential toxicity from 6-MMPR. Initial short-term reports on efficacy and toxicity of TG were promising. In 2003, however, a study of IBD patients reported the occurrence of NRH in 16 out of 26 (62%) biopsies taken from 111 patients treated with TG. The authors concluded that the complication was idiosyncratic and that
TG should no longer be considered as a therapeutic option in IBD patients since data on progression to (complicated) non-cirrhotic portal hypertension or, alternatively, reversibility of NRH was lacking. On the other hand, it should be noted that most patients were pre-treated with AZA or MP and in this study 40% of patients had signs of hepatotoxicity during this preceding therapy. Moreover, the dosage of TG administered in this study was not reported accurately, whilst the observed median 6-TGN level was approximately 1250 pmol/8x10⁸ RBC (almost three times the upper limit of the putative therapeutic range used with AZA and MP therapy). Following this article, several other research groups published their data on TG therapy and the prevalence of NRH (table 2)¹ⁱ,¹⁶²,¹⁶⁵-¹⁶⁸. The alarmingly high prevalence rate of NRH of 62% was not observed in any other study. These subsequent studies appear to show a dose-dependent NRH effect with no NRH observed in the patient groups treated with a maximum of 20mg of TG daily with a corresponding median 6-TGN level of approximately 600 pmol/8x10⁸ RBC¹⁶⁷,¹⁶⁸. In patients treated with higher dosages (40 to 80mg per day), NRH was observed in 0 to 27% of liver biopsies. The reversibility of NRH and its potential complications have been studied by measurement of the hepatic venous pressure gradient¹⁶⁶. It was demonstrated that discontinuation of TG therapy attenuates portal hypertension reducing the risk from this complications. As therapy with AZA and MP has also been associated with the development of NRH, induction of NRH might be a drug-class effect (related to thiopurines in general), instead of solely a dose related TG effect. Based on the aforementioned data, the administration of low-dose TG in IBD patients failing classical thiopurines should be reconsidered, but further studies on the (hepato-)toxicity and efficacy profile of TG are awaited to allow for more definitive recommendations for its potential use.

Table 2. Nodular regenerative hyperplasia of the liver during tioguanine therap

<table>
<thead>
<tr>
<th>Dosage of TG</th>
<th>6-TGN level</th>
<th>Observed NRH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>unknown</td>
<td>1230 (530-2310)</td>
<td>62% (16/26)</td>
<td>[9]</td>
</tr>
<tr>
<td>40 - 80 mg/day</td>
<td>unknown</td>
<td>18% (8/45)</td>
<td>[10]</td>
</tr>
<tr>
<td>40 mg/day</td>
<td>807 (105-2545)</td>
<td>0% (0/11)</td>
<td>[160]</td>
</tr>
<tr>
<td>20-40 mg/day</td>
<td>unknown</td>
<td>27% (16/60)</td>
<td>[163]</td>
</tr>
<tr>
<td>20 mg/day</td>
<td>564 (SD 278)</td>
<td>0% (0/28)</td>
<td>[166]</td>
</tr>
<tr>
<td>20 mg/day</td>
<td>802 (106-1092)</td>
<td>0% (0/13)</td>
<td>[165]</td>
</tr>
</tbody>
</table>

TG, tioguanine; 6-TGN, 6-thioguanine nucleotides (expressed in pmol/8x10⁸ RBC), presented as median with range or mean with standard deviation (SD); NRH, nodular regenerative hyperplasia.

**CLINICAL RECOMMENDATIONS**

**Drug monitoring**

The safety and efficacy of thiopurine therapy was historically, and still is, monitored by means of a full blood count, liver function tests and a clinical assessment. In recent years, evidence has emerged that thiopurine pharmacogenetics and metabolite monitoring may improve clinical outcomes and reduce the occurrence of adverse events during thiopurine
therapy. Several modalities to guide and monitor thiopurine therapy have been developed and proposed for use in clinical practice (table 3). Unfortunately, the clinical benefits of each of these monitoring methods are limited and further research is necessary to justify widespread implementation of these into clinical practice. Among these, the assessment of thiopurine S-methyl transferase (TPMT) activity and measurement of thiopurine metabolite concentrations are the most extensively studied. Measuring TPMT activity prior to the initiation of thiopurines, either by genotype or phenotype, may identify the one out of 250 patients with very low TPMT activity in Caucasians who may be judiciously prescribed ultra-low thiopurine doses with careful monitoring. Those with intermediate TPMT activity (5-10 nmol 6-methyl mercaptopurine x g⁻¹ Hb x h⁻¹) will require 33% to 50% of a standard thiopurine dose as they also are less likely to tolerate a standard dose of thiopurines. Since screening for the most common defective TPMT alleles will not prevent all cases of thiopurine induced myelotoxicity, blood monitoring remains mandatory. In this respect, phenotype measurement may be a more reliable, although imperfect, predictor of thiopurine associated myelotoxicity. It is worth noting that recent blood transfusions will affect measurement of TPMT activity (in red blood cells) and that ethnicity affects the frequency of the common defective TPMT alleles. Although not supported by firm evidence, TPMT screening of phenotype in particular, may allow less frequent blood monitoring in Caucasian patients with normal TPMT activity. It will also allow up front correct dosing of thiopurines so reducing time to clinical pharmacodynamic response. Studying the cost-effectiveness of TPMT screening is therefore of much interest and potential clinical value. While some pharmacoeconomic modelling studies show that TPMT testing is cost-effective, prospective studies, performed in a variety of different health delivery environments, are needed to establish properly informed guidelines.

Interpretation of metabolite profiles and recommendations for dose adjustments are shown in table 4. Apart from the above mentioned limitation that metabolite measurement in RBC is a surrogate for the target cells, other reasons may contribute to the variable utility of therapeutic drug monitoring. First, due to technical limitations and variations in metabolite assays we may not be measuring all the relevant metabolites or be measuring these with sufficient specificity or reliability. Second, IBD represents an extremely heterogeneous group of patients, which is exemplified by some patients who do not achieve clinical remission despite adequate thiopurine therapy with ‘adequate’ 6-TGN concentrations; these patients are thiopurine refractory. In addition, some patients show a clinical response while 6-TGN concentrations are sub-therapeutic.

The presence of these two groups of patients hampers the interpretation of studies that aim to assess a relation between drug efficacy and clinical outcome. In addition to these difficulties with interpretation of dose-clinical response, limited access in hospitals in many parts of the world and the financial burden of testing compels other strategies to monitor and optimize thiopurine treatment. In the case of TG, no strong recommendations on the determination of 6-TGN concentration can be put forward as evidence is lacking. However, we suggest reducing the TG dose if 6-TGN concentrations exceed 1200 pmol/8x10⁸ RBC.
<table>
<thead>
<tr>
<th>Modality</th>
<th>Predicts</th>
<th>Method</th>
<th>Source</th>
<th>Interpretation</th>
<th>Costs (€*)</th>
<th>References</th>
</tr>
</thead>
</table>
| TPMT genotype     | TPMT enzyme activity                    | PCR    | PBMC   | • At least 28 variant alleles of the TPMT gene have been identified. In the Caucasian population, three variant alleles appear most frequently: TPMT*2, *3A, *3C. These three alleles are usually used for screening.  
• Heterozygosity of one variant allele with one wild-type allele warrants a 50-66% dose reduction of AZA or MP. Homozygosity of these variant alleles precludes the use of thiopurines or extremely low dosing of thiopurines could be contemplated.  
• Disadvantage: genotype-phenotype concordance if only screened for three variant alleles is poor.                                                                                     | 50-250,-   | [61,80,227]|
| TPMT enzyme       | TPMT enzyme activity                    | HPLC   | RBC    | • TPMT activity in a Caucasian population is distributed in a trimodal manner: low (0.6%), intermediate (10.2%) and normal/high (89.2%/1.8%)  
TPMT activity.  
• Pitfalls: determined TPMT activity may not reflect the patient's true activity in case a patient has recently received blood transfusions. TPMT activity may also be altered by co-medications or thiopurine therapy itself. | 50-250,-   | [56,64,227]|
| 6-TGN             | Pharmacological response, non-compliance in combination with 6-MMPR | HPLC   | RBC    | • Measured in RBC, their concentration is a surrogate marker for pharmacodynamic response in leukocytes.  
• Reference ranges are method specific and may depend on other clinical factors.  
• Patients with [6-TGN] 230-400 are more likely to show a clinical response.  
• Patients with [6-TGN] > 400 are more likely to develop myelotoxicity.                                                                                                                        | 100-200,-  | [121,131,140,227]|
| 6-MMPR            | Pharmacological response, non-compliance in combination with 6-TGN | HPLC   | RBC    | • Measured in RBC, their concentration in relation to [6-TGN] provides information about thiopurine metabolism preferences.  
• Patients with high [6-MMPR] are more likely to develop hepatotoxicity.                                                                                                                    | 0,-        | [228]      |
| Algorithms        | Clinical response, non-compliance [6-TGN] and [6-MMPR] low and shunting ([6-MMPR]/[6-TGN]>20) | Mathematical Computer modelling from FBC and biochemistry parameters | • Different algorithms use routine haematological and biochemical parameters to predict clinical response, non-compliance or the presence of a shunting metabolism (toward 6-MMPR).  
• The outcomes non-compliance and presence of a shunting metabolism were based on [6-TGN] and [6-MMPR] values.  
• Pitfall: with respect to the outcome clinical response, the algorithm does not provide any information about the pharmacological response to thiopurines. | 0,-        | [229]      |
| MCV               | [6-TGN]                                 | Mathematical Blood | • MCV increase is an independent correlator of RBC [6-TGN]. But sensitivity and specificity to predict if [6-TGN] lies within the reference range are limited.                          | 0,-        | [229]      |

*One euro is approximately 1.3 U.S. dollar. TPMT, thiopurine S-methyl transferase; 6-TGN, 6-thioguanine nucleotides; 6-MMPR, 6-methyl mercaptopurine ribonucleotides; MCV, mean cellular volume; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; PBMC, peripheral blood mononuclear cells; RBC, red blood cells; FBC, full blood count. [6-TGN] and [6-MMPR] are expressed in pmol/8x10⁸ RBC.
Thiopurine therapy during clinical remission

As thiopurine therapy is associated with a wide range of adverse events, more data are required to determine the optimal duration of therapy, particularly for patients in remission. Over the years, several thiopurine withdrawal trials have been carried out, some of which were placebo controlled. Overall, they show that, irrespective of the duration of remission, withdrawing thiopurine therapy increases the risk of relapse, both in Crohn’s disease and ulcerative colitis. There is some evidence that this is a dose-related effect. In those patients who withdrew thiopurine therapy, male gender, younger age, duration of remission less than four years, CRP >20mg/L, neutrophil count >4.0x10⁹/L, and hemoglobin <7.4 mmol/l were independently associated with an increased risk of relapse. Given these results that continuation may be favorable in the majority of patients, there remains a minority who needlessly continue thiopurine therapy and are exposed to the associated risks. Accordingly, the identification of patients who, despite cessation of thiopurine therapy, will be at a low risk of relapse is of particular interest.

Thiopurine therapy in the postoperative setting

Postoperative recurrence is a major concern in Crohn’s disease because disease recurrence of disease activity occurs in almost 50% of all patients within 10 years after the first resection. Without drug therapy, the clinical recurrence rate is about 20%-25% after the first year. Azathioprine and MP are more effective in preventing clinical recurrence at one year and two years after surgery as compared with placebo or mesalazine. In addition, at one year, thiopurines are more effective than placebo or mesalazine in preventing severe endoscopic

<table>
<thead>
<tr>
<th>6-TGN</th>
<th>6-MMPR</th>
<th>Non-response</th>
<th>Dose dependent adverse event</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;=230</td>
<td>&lt;=5700</td>
<td>Non-compliance</td>
<td>-</td>
<td>Gain compliance</td>
</tr>
<tr>
<td>&lt;230</td>
<td>&lt;5700</td>
<td>Under dosing</td>
<td>-</td>
<td>Increase dose (+50mg AZA; +25mg MP)</td>
</tr>
<tr>
<td>230-400</td>
<td>&lt;5700</td>
<td>Possible therapy resistance</td>
<td>-</td>
<td>Increase dose or change drug therapy</td>
</tr>
<tr>
<td>&gt;400</td>
<td>&lt;5700</td>
<td>Therapy resistance</td>
<td>Increased risk of myelosuppression</td>
<td>Change drug therapy</td>
</tr>
<tr>
<td>&lt;230</td>
<td>&gt;=5700</td>
<td>Shunting</td>
<td>Increased risk of hepatotoxicity</td>
<td>Consider allopurinol† co-treatment to increase 6-TGN and decrease the risk of hepatotoxicity</td>
</tr>
<tr>
<td>230-400</td>
<td>&gt;=5700</td>
<td>Therapy resistance</td>
<td>Increased risk of hepatotoxicity</td>
<td>Change drug therapy; consider allopurinol† co-treatment to decrease risk of hepatotoxicity</td>
</tr>
<tr>
<td>&gt;400</td>
<td>&gt;=5700</td>
<td>Therapy resistance</td>
<td>Increased risk of myelosuppression and hepatotoxicity</td>
<td>Change drug therapy; consider allopurinol† co-treatment</td>
</tr>
</tbody>
</table>

6-TGN, 6-thioguanine nucleotides; 6-MMPR, 6-methyl mercaptopurine ribonucleotides; 6-TGN and 6-MMPR concentrations are expressed in pmol/8x10⁸ RBC. † allopurinol co-treatment (100mg daily) requires a dose reduction of azathioprine or mercaptopurine up to approximately 25-33% of the original dose.231
Nitroimidazole antibiotics, including metronidazole and ornidazole, also effectively prevent postoperative recurrence. However, these are only effective in the short term and are less well tolerated.

It is questionable whether all patients need postoperative thiopurine therapy. In a European consensus paper prophylactic treatment is recommended after small intestinal resection. The presence of risk factors for early postoperative recurrence mandates thiopurines as therapy of choice in this consensus paper, although anti-TNFα therapy may be preferable to prevent severe recurrence. Aside from absence of prophylactic treatment, ongoing cigarette smoking, prior intestinal surgery, penetrating disease behavior, perianal disease and extensive small bowel resection are predictors of early postoperative recurrence after ileocolonic resection. In patients without any of these predictors one may consider high dose mesalazine as prophylactic treatment, but still assess the risk of clinical recurrence by assessing endoscopic recurrence within six to 12 months of surgery, with a view to initiating thiopurine therapy if severe endoscopic lesions are present (Rutgeerts score ≥12). Prophylactic treatment is recommended to start within two weeks of surgery, although there are no data showing that this approach is superior to delayed treatment.

It is yet unknown, for what time CD patients in the postoperative setting should continue thiopurine therapy if both clinical and endoscopic remission are maintained. Based on the aforementioned results from thiopurine withdrawal trials, including IBD patients who had not underwent any bowel resection, we recommend to continue thiopurine therapy as long as possible in the postoperative setting. More research is needed to support this.

CONCLUSIONS

Thiopurine therapy remains central to IBD treatment. Over the years, knowledge of their pharmacological properties, clinical utility and toxicity profile has grown notably leading to a safer and more efficacious use of thiopurine derivatives. Yet, many pertinent questions remain to be answered. Future studies must aim to improve our understanding of individual pharmacological responses to optimize and exploit the therapeutic potential and reduce adverse events of these drugs.
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PHARMACOLOGICAL CONSIDERATIONS OF THIOPURINES IN THE TREATMENT OF INFLAMMATORY BOWEL DISEASE


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