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Thiopurines in inflammatory bowel disease

New strategies for optimization of pharmacotherapy

Luc JJ Derijks

Thiopurines in inflammatory bowel disease

New strategies for optimization of pharmacotherapy

ACADEMISCH PROEFSCHRIFT

Ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van Rector Magnificus
Prof. Mr. P.F. van der Heijden
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de Aula der Universiteit op
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*Intellectuals solve problems,
geniuses prevent them.*

Albert Einstein (1879-1955)

*voor mijn ouders
voor Judith*

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Part I

General introduction

Chapter 1

Thiopurines in inflammatory bowel disease, state of the art

LJJ Derijks, LPL Gilissen, PM Hooymans, DW Hommes

Submitted for publication

Introduction

Thiopurines are widely used in the treatment of inflammatory bowel disease (IBD). However, in clinical practice, azathioprine (AZA) or 6-mercaptopurine (6-MP) are not effective in one third of patients and up to one fifth of patients discontinues thiopurine therapy due to adverse events. Recent insights in thiopurine pharmacology and pharmacogenetics contributed to the development of new strategies to improve pharmacotherapy. In this review of literature thiopurine efficacy, toxicity, pharmacology, pharmacogenetics, interactions and new strategies for optimization of pharmacotherapy in particular are described.

Efficacy

Thiopurines have proven efficacy in IBD [1-3]. In clinical practice, in 68% of IBD patients the initial therapeutic goal (i.e. mucosal healing, elimination of steroids, healing of internal fistulas or abscesses, pain relief, etc) is achieved [4] and after initial response efficacy is reasonably well sustained with remission rates of 95%, 69% and 55% after 1, 3 and 5 years respectively [5].

Both AZA and 6-MP have proven efficacy in induction of remission in active Crohn's disease (CD) with odds ratios (OR) up to 3.1 compared to placebo [2, 6]. The OR of response increases after 17 weeks, suggesting there is a minimum length of time for trial of thiopurine therapy [6]. Thereby, the thiopurines have a considerable steroid-sparing effect and the combination of AZA with prednisolone is superior to treatment with prednisolone alone [2, 7].

AZA and 6-MP seem to be effective in maintaining remission of quiescent CD patients that are steroid-dependent or -refractory or in CD-patients who have finished with surgical treatment with a number needed to treat (NNT) of 7 [2, 8-11], though not everybody is convinced [12]. Also, in the prevention of exacerbations, the addition of 6-MP to a regimen of steroids significantly lessens the need for prednisone [13, 14]. There is debate for how long thiopurines should be given to CD-patients in prolonged remission. Some question the use of potential toxic immunosuppressants for longer than four years [15], whereas others demonstrate that their efficacy is reasonably well sustained over a period of five years [5].

AZA and 6-MP in combination with other immunosuppressants like cyclosporine, tacrolimus, methotrexate, thalidomide or infliximab have proven efficacy in CD perianal fistulas with prolonged fistula closure in 30-40% of cases [16-19]. Also, both thiopurines help to prevent the recurrence of CD after surgery, especially in high risk patients, with a NNT of 4 in two years to prevent one clinical relapse [20-23].

Although less pronounced than in CD populations, both AZA and 6-MP are effective in inducing remission in ulcerative colitis (UC) with remission rates up to 70% [1, 24,

25]. However, scarce but conflicting data have been published, demonstrating AZA had no effect at all in achieving remission [26].

The role of thiopurines in maintaining remission of UC is less controversial [1]. AZA maintenance treatment in UC is beneficial for at least two years after an initial response and significantly lowers the proportion of relapses [26, 27]. Moreover, combination therapy is more effective than treatment with steroids [1, 14, 28] or 5-aminosalicylic acids (5-ASA) alone [29]. Also, the combination of thiopurines and oral cyclosporine has proven to be safe and effective for maintenance of intravenous cyclosporine-induced remission of severe steroid-refractory UC [30, 31], though monotherapy with thiopurines may be sufficient [32].

Toxicity

In IBD patients treated with thiopurines, up to 20% has to discontinue drug therapy due to the occurrence of adverse events [33-35]. The adverse events of thiopurines can be divided into dose-dependent, pharmacologically explainable events (type A) on one hand and dose-independent, hypersensitivity reactions (type B) on the other. Type A adverse events, which often reveal themselves in a subsequent stage, are largely associated with the formation of potentially toxic metabolites and include general malaise and nausea (11%), infectious complications (7.4%), hepatitis (0.3-1.3%) and myelosuppression (1.4-5%) [2, 34-36]. The reported frequency of the leukopenia may be as high as 11%, depending on the definition of leukopenia and the dose prescribed [37]. Type B reactions (2%) often occur within three to four weeks after start of treatment and result in immune-mediated symptoms like fever, rash and arthralgia [38, 39]. Also, pancreatitis (1.4-3.3%) is thought to be an idiosyncratic reaction [6, 34].

Although increased frequencies of malignancies are reported in other patient populations on thiopurines, this seems not to be the case in IBD patients [40]. Moreover, in a decision analysis it was recently concluded that the benefits of thiopurines outweigh the potential risk of lymphoma [41]. Also, the use of thiopurines before or during pregnancy appears to be safe as there is no statistical difference in abortions, congenital malformations or neoplasia among IBD patients on thiopurines compared with healthy controls [42].

Pharmacology

Pharmacokinetics

Neither AZA nor 6-MP has intrinsic activity, hence both have to undergo extensive metabolic transformations. AZA is a pro-drug that is rapidly and almost completely (88%) converted to 6-MP and methylnitroimidazole (figure 1, cleavage site 1) by a non-enzymatic reaction in the liver. The latter causes intolerance in a considerable

group of patients of whom 75% successfully switches to 6-MP [43], but may have immunosuppressive properties of its own [44]. The remaining 12% of AZA yields hypoxanthine and methylnitrothioimidazole (figure 1, cleavage site 2) [45].

These non-enzymatic conversions are aided by glutathione or other endogenous sulphhydryl-containing proteins [46, 47]. Smoking and higher age lower glutathione levels [48] and may cause larger variability in the amount of 6-MP originating from AZA [49]. The absorption of AZA is incomplete and variable resulting in a bioavailability of 16-72% [46]. The bioavailability of 6-MP originating from AZA is approximately 60% compared to only 5-37% when 6-MP is administered as such, mainly as a result of extensive catabolic conversion of the latter to 6-thiouric acid (6-TUA) in the bowel and liver by xanthine oxidase (XO) [50]. The molecular weight of 6-MP ($M_w = 152.18$ g/mol) is 55% of the molecular weight of AZA ($M_w = 277.27$ g/mol), resulting in a conversion factor of 2.08 ($1/0.55 \times 1/0.88$) when converting 6-MP to an equivalent pharmaceutical dose of AZA assuming 100% bioavailability [51]. The plasma half-life of 6-MP is very short, at the most 2 hours.

Following intracellular uptake, 6-MP is then further metabolized by three enzymes (figure 2) two of which are catabolic, XO and thiopurine S-methyltransferase (TPMT), and one is anabolic, hypoxanthine phosphoribosyl transferase (HPRT) [45]. XO metabolizes 6-MP to 6-TUA, whereas TPMT methylates 6-MP to 6-methylmercaptopurine (6-MMP).

HPRT carries out the first anabolic step to produce 6-thioinosine monophosphate (6-TIMP), which is further transformed by inosine monophosphate dehydrogenase (IMPD) in a rate-limiting step into 6-thioxanthosine monophosphate (6-TXMP), which is ultimately metabolized to 6-thioguanine monophosphate (6-TGTP), diphosphate (6-TGDP) and triphosphate (6-TGTP), together the so-called 6-thioguaninenucleotides (6-TGN). The 6-TGN have a half-life of approximately 5 days with a large variability of 3-13 days [46, 52]. 6-TIMP can alternatively be methylated by TPMT, yielding 6-methylthioinosine monophosphate (6-MTIMP), diphosphate (6-MTIDP) and triphosphate (6-MTITP), the so-called 6-methylmercaptopurine ribonucleotides (6-MMPR). Finally, it is hypothesized that 6-TIMP successively is converted into 6-thioinosine diphosphate

Figure 1. Chemical structures of the thiopurines. For azathioprine, cleavage sites of the non-enzymatic reaction in the liver are depicted. Cleavage site 1 yields 6-mercaptopurine and methylnitroimidazole while cleavage site 2 yields hypoxanthine and methylnitrothioimidazole.

Figure 2. Proposed thiopurine metabolism. AZA, azathioprine; 6-MP, 6-mercaptopurine; 6-MMP, 6-methylmercaptopurine; 6-TUA, 6-thiouric acid; 6-MTIMP, 6-methylthioinosine monophosphate; 6-MTIDP, 6-methylthioinosine diphosphate; 6-MTITP, 6-methylthioinosine triphosphate; 6-TIMP, 6-thioinosine monophosphate; 6-TIDP, 6-thioinosine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-TG, 6-thioguanine; 6-MTG, 6-methylthioguanine; XO, xanthine oxidase; TPMT, thiopurine S-methyltransferase; HPRT, hypoxanthine phosphoribosyl transferase; IMPD, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase; MPK, monophosphate kinase; DPK, diphosphate kinase; ITPase, inosine triphosphate pyrophosphatase. 6-MTIMP, 6-MTIDP and 6-MTITP together form the 6-methylmercaptopurine ribonucleotides (6-MMPR). 6-TGMP, 6-TGDP and 6-TGTP together form the 6-thioguaninenucleotides (6-TGN). In therapeutic drug monitoring, a 6-MMP level consist of the sum of 6-MMP, 6-MTIMP, 6-MTIDP and 6-MTITP levels, while a 6-TG level consist of the sum of 6-TGMP, 6-TGDP, 6-TGTP (if AZA or 6-MP is administered) or 6-TG, 6-TGMP, 6-TGDP, 6-TGTP (if 6-TG is administered as such) levels.

(6-TIDP) and triphosphate (6-TITP) to form 6-TIMP once again by the enzyme inosine triphosphate pyrophosphatase (ITPase).

In contrast to AZA and 6-MP, pharmacokinetics (metabolism in particular) of 6-thioguanine (6-TG) is much less complicated. The absorption of oral 6-TG is incomplete and variable, resulting in a bioavailability of 14-46% [50]. Plasma 6-TG concentrations may range up to thirty-fold [53] and become undetectable after 6 hours as it is rapidly transported into the cell [54, 55]. HPRT directly converts 6-TG to the 6-TGN, whereas the competing TPMT and XO yield 6-methylthioguanine (6-MTG) and 6-TU respectively [56].

Pharmacodynamics

In recent years, the molecular mechanisms of action of the thiopurines have been elucidated at least in part. The 6-TGN (figure 3), as a result of their structural similarity to the endogenous purine-base guanine, are incorporated into DNA (figure 4) of leukocytes as fraudulent bases, resulting in strand breakage and subsequently immunosuppression [45, 57]. Also, one of the 6-TGN in particular, 6-TGTP, is considered to contribute to the immunosuppressive effects due to inhibition of Rac1 upon CD28 co-stimulation, inducing T-cell apoptosis [58]. Rac1 is a small GTPase and plays a role in inhibiting T-cell apoptosis. 6-TGTP binds to Rac1 instead of GTP, thereby suppressing the activation of Rac1 target genes like mitogen-activated protein kinase, NF- κ B and bcl-xL.

Also, the 6-MMPR contribute to the antiproliferative properties of the thiopurines, probably through inhibition of de novo purine synthesis [59]. Furthermore, as mentioned before, AZA has an immunosuppressive effect additional to that attributable to 6-MP alone and it is proposed that this is associated with an action of the methyl-nitroimidazolyl substituent by a so far unsolved mechanism [44]. In addition to this, other mechanisms may contribute to the immunosuppressive effects of the thiopurines, such as interference with the function of similar endogenous molecules like ATP and GTP, which play an important role as carriers of energy and cellular second messengers [60].

It is obvious, that the immunosuppressive properties of the thiopurines are not attributable to one unique mechanism, but are based on multiple and complex mechanisms. To date however, it is unclear to what extent these mechanisms contribute to the overall effect of the thiopurines.

Figure 3. Chemical structures of the 6-thioguaninenucleotides (6-TGN). 6-TGMP, 6-thioguanine monophosphate, 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate.

Figure 4. Chemical structure of DNA. The 6-thioguaninenucleotides exhibit strong structural similarity to the endogenous purine-base guanine. They possess a thiole-group instead of an oxygen-atom, resulting in strand breakage when they are incorporated into DNA as fraudulent bases.

Pharmacogenetics

The observed interindividual differences in therapeutic response or toxicity are partly explained by the variable formation of active metabolites due to genetic polymorphisms of the genes encoding crucial enzymes in thiopurine metabolism. These enzymes include TPMT, XO, HPRT and ITPase.

TPMT

One of the most intensively studied genetic polymorphisms is that of TPMT, though neither its biological function nor the endogenous substrates are known [61]. In thiopurine-treated patients, TPMT determines the delicate balance between the 6-MMPR and the 6-TGN. The gene encoding TPMT is located upon chromosome 6 (6p22.3) and contains 10 exons. To date, 2 wild-type alleles (TPMT*1 and *1S) and 20 mutant alleles (TPMT*2, *3A, *3B, *3C, *3D, *4, *5, *6, *7, *8, *9, *10, *11, *12, *13, *14, *15, *16, *17, *18) responsible for TPMT deficiency have been described (figure 5a+b) [62, 63]. These alleles are characterized by one or more single nucleotide polymorphisms (SNPs) in the open reading frame (ORF) sequences of the TPMT gene. Besides, several intronic mutations and mutations outside the ORF exist. Recently, a variable num-

ber tandem repeats (VNTR) within the TPMT promoter region of the TPMT-gene has also been reported to modulate levels of TPMT activity [64, 65]. Enhanced degradation of TPMT proteins encoded by certain mutant alleles has been proposed as mechanisms for lower TPMT protein and catalytic activity: whereas the degradation half-life of TPMT*1 is 18 hours, TPMT*2 and TPMT*3A have a strongly decreased half-life of 15 minutes [66]. The distribution of TPMT mutant alleles differs significantly among ethnic populations. TPMT*3A (3.2-5.7%) is the most occurring mutant allele in Caucasian populations, followed by TPMT*2 (0.2-0.5%) and TPMT*3C (0.2-0.8%) accounting for the vast majority (>95%) of mutant alleles [60-62, 67-73]. In Asians and African populations however, TPMT*3C is the most frequent mutant allele [74-76].

There is high correlation between TPMT geno- and phenotype [36, 63]. The resulting frequency distribution of TPMT activity in Caucasian populations is trimodal: approximately 89% of the population are homozygous for the wild-type allele (homozygous TPMT^{tt}) and consequently have high enzyme activity (13.50 ± 1.86 U/ml RBC), 11% have inherited one wild type allele and one mutant allele (heterozygous TPMT^{tt}/TPMT^l) and have intermediate levels of enzyme activity (7.20 ± 1.08 U/ml RBC), while 1 in 300 subjects has 2 mutant alleles (homozygous TPMT^l) and low or no detectable enzyme activity at all [60, 77]. TPMT activity is high in children compared to adults, among adults higher in men than in women and higher in smokers than in non-smokers [63, 78, 79]. Also, TPMT activity significantly increases during thiopurine treatment as a result of enzyme induction [45].

XO

XO is a cytoplasmic enzyme that oxidizes endogenous substrates such as hypoxanthine and xanthine, but also the exogenous 6-MP. Its activity is particularly high in the intestinal mucosa and liver [80], resulting in a substantial reduction of 6-MP bioavailability. On the other hand, oxidized thiopurine metabolites may inhibit TPMT, resulting in higher levels of the active 6-TGN [81]. There exists 4-fold interindividual variation in hepatic XO activity and the presence of a subgroup of patients with low activity is suspected. XO activity is approximately 20% higher in men than in women [82]. The molecular basis of these differences remains unclear [45].

HPRT

Only limited information exists on the range of HPRT activity. What is known however, is that HPRT is completely deficient in subjects with the very rare (1:100,000) Lesch-Nyhan syndrome characterized by self-mutilating behaviors such as lip biting and head banging [45]. Thiopurines are not cytotoxic in Lesch-Nyhan patients and SNPs in the HPRT-gene appear to be the molecular basis of this syndrome [83]. Moreover, HPRT activity increases during long-term thiopurine treatment [45].

ITPase

ITPase catalyzes the pyrophosphohydrolysis of inosine triphosphate (ITP) to inosine

TPMT wild type

TPMT mutant alleles (1)

Figure 5a. Thiopurine S-methyltransferase (TPMT) genotypes (1). The black and white boxes represent translated and untranslated exons respectively. The grey boxes represent translated exons on mutant alleles containing a single nucleotide polymorphism (SNP); the SNP is captioned below the relevant exon.

monophosphate (IMP) and deficiency leads to abnormal accumulation of ITP, which is not known to be associated with any defined pathology [84]. In ITPase-deficient patients treated with AZA or 6-MP, accumulation of 6-TITP is suggested [85]. The gene encoding ITPase, ITPA, is located on chromosome 20 and 5 SNPs have been identified so far, of which three (G138A, G561A, G708A) are silent and two (C94A, IVS2 + A21C)

TPMT mutant alleles (2)

Intronic- and outside ORF mutations

Figure 5b. Thiopurine S-methyltransferase (TPMT) genotypes (2). The black and white boxes represent translated and untranslated exons respectively. The grey boxes represent translated exons on mutant alleles containing a single nucleotide polymorphism (SNP); the SNP is captioned below the relevant exon. ORF, open reading frame.

are associated with decreased ITPase activity (figure 6) [86]. C94A is the most frequently occurring SNP. Homozygotes for the C94A missense mutation have no enzyme activity at all and IVS2+A21C homozygotes have approximately 60% of normal ITPase activity, but the incidence is unknown. It is estimated that approximately 6% of the population are C94A heterozygotes and they possess 22.5% of normal enzyme activity [86].

Interactions

A number of coadministered drugs may potentially influence thiopurine metabolism and consequently has to be taken into account. In vitro studies have demonstrated 5-aminosalicylic acid (5-ASA) compounds (i.e. balsalazide, olsalazine and sulphasalazine) to be potent TPMT inhibitors [87-89]. In vivo, 6-TGN concentrations are significantly higher when thiopurines are combined with 5-ASA. The observed higher 6-TGN levels can not solely be explained by TPMT inhibition and other yet unknown interaction mechanisms may exist [90]. Consequently, a higher frequency of leukopenia is observed in patients using this combination [87, 91]. Other frequently prescribed TPMT inhibitors include acetylsalicylic acid [92] and furosemide [93]. Allopurinol potently inhibits XO and a dose-reduction to 25-33% of standard daily dosages of AZA or 6-MP is recommended when combined to prevent serious myelotoxicity [50, 94]. Mycophenolate inhibits IMPD, theoretically reducing the conversion of 6-TIMP to 6-TXMP and 6-TGN consequently [36].

New strategies

In the past 10-20 years, knowledge of both thiopurine pharmacology and pharmacogenetics has been extended dramatically and used to develop new strategies to improve efficacy and reduce toxicity. These strategies include therapeutic drug monitoring (TDM) of thiopurine metabolites, geno- or phenotyping crucial enzymes in thiopurine metabolism like TPMT and ITPase and the use of 6-TG as such. All strategies and their potential are discussed.

Therapeutic Drug Monitoring

The active metabolites of the thiopurines, 6-TGN and 6-MMPR, can be quantified in the human red blood cell (RBC) by a reversed-phase high-performance liquid chromatographic (HPLC) assay with UV-detection developed by Lennard and colleagues [95, 96]. In brief, for measurement of intracellular thionucleotides the free base is obtained by acid hydrolysis of the nucleotide back to the purine. The resulting purines are extracted from the biological matrix by forming a phenylmercury adduct into toluene. During back-extraction with hydrochloric acid the adduct is split and the free thiopurine liberated into the acid layer once again. RBCs are a good surrogate matrix because they are easily obtained, exist in sufficient numbers and contain concentrations of 6-TGN that reflect concentrations of 6-TGN in the less accessible putative target tissues, the leukocytes [97]. Various other chromatographic methods for the same purpose have been developed ever since, but considerable differences in 6-TGN concentrations are observed when comparing these methods, probably attributable to the extent of nucleotide hydrolysis, extraction procedure, chromatographic conditions and method of detection [98, 99]. Direct comparison of several methods with the

Figure 6. Location of the five single nucleotide polymorphisms (SNPs) on the inosine triphosphate pyrophosphatase gene (ITPA). The black and white boxes represent translated and untranslated exons respectively. G138A, G561A and G708A are silent SNPs, while C94A and IVS2+A21C are missense mutations associated with decreased ITPase activity.

Lennard-method showed that metabolite concentrations are 2.6-fold higher in the Dervieux-method [100], 1.4-fold higher when both slightly modified [101] and 1.6-fold lower when determined by the Erdmann-method [102]. The Lennard-method has found the greatest application in clinical studies yet and has served as the basis for the establishment of treatment-related therapeutic ranges for thiopurine therapy [99].

In one of the first clinical studies in which thiopurine metabolites were assayed, a correlation was found between 6-TGN levels and therapeutic efficacy on one hand and between 6-MMPR levels and toxicity on the other [103]. In another study with a pediatric population specific metabolite target levels were proposed: the frequency of therapeutic response increased at 6-TGN levels above 235 picomoles/ 8×10^8 RBC (OR: 5.0) and hepatotoxicity correlated with 6-MMPR levels above 5700 picomoles/ 8×10^8 RBC, the risk increasing 3-fold [104]. In a subpopulation with leukopenia a mean 6-TGN level of 490 picomoles/ 8×10^8 RBC was measured [104].

Since then, in large cohorts of both pediatric and adult populations, conflicting data have been published. A comparable correlation between metabolite concentrations and efficacy and toxicity was demonstrated by several studies. In a study with 82 adult IBD patients, 6-TGN concentrations above 250 picomoles/ 8×10^8 RBC correlated with treatment efficacy [105]. In another study with 60 adult IBD patients, 6-TGN levels also were associated with clinical response and though there was a fair amount of overlap, the maximal differentiation between responders and non-responders was seen at 6-TGN levels above 260 picomoles/ 8×10^8 RBC [106]. In yet another study with 131 adult IBD patients with evaluable 6-TGN levels over a 2 year period, patients who remained in remission had significantly higher mean 6-TGN levels than those patients who developed active disease. 6-MMPR levels did neither correlate with drug efficacy nor toxicity [49]. Similar results were found in a study with 55 IBD patients: 6-TGN level but not drug dose was related to disease activity [107]. Finally, an inverse relationship between 6-MMPR: 6-TGN ratio and therapeutic efficacy has been described and a ratio of 11 has been suggested as a cut-off point above which the frequency of response is diminished [108, 109]. Other immunosuppressive therapy should be considered in this case.

These results are contradicted by the findings of other studies that showed no or weak correlation between metabolite concentrations and efficacy or toxicity. In 28 patients with CD, there was a broad overlap in 6-TGN concentrations between patients in remission or not and responders to AZA/6-MP therapy or not [110]. Also, 6-TGN concentrations did not correlate with disease activity in a cohort of 170 IBD patients [111]. In another study with 101 pediatric IBD patients, 58% of the patients in remission had 6-TGN levels less than the 235 picomoles/ 8×10^8 RBC proposed by Dubinsky and colleagues [112]. Furthermore, poor correlation between metabolite levels and efficacy or toxicity was found in a study with 74 eligible IBD patients: 38% of patients with active disease had a 6-TGN level above 235 picomoles/ 8×10^8 RBC and no hepatotoxicity was observed despite 6-MMP levels above 5700 picomoles/ 8×10^8 RBC in 12.2% of patients [113].

Currently, it is much debated whether assessment of metabolite concentrations is clinically useful in predicting both drug efficacy and toxicity [114, 115]. These controversies, at least in part, result from the lack of proper pharmacokinetic data in IBD patients. In the near future, these data need to be collected in prospective pharmacokinetic trials to elucidate this matter. Unquestioned however, is the fact that therapeutic drug monitoring is the only way to reveal non-compliance [116, 117].

Geno- and phenotyping

Another strategy that may contribute to a better pharmacotherapy of IBD with thiopurines is genotyping genes encoding key enzymes in their metabolism, such as TPMT. Reliable polymerase chain reaction (PCR)-based methods have been developed for detecting the major inactivating mutations at the human TPMT locus [61, 69, 118]. In brief, leukocyte DNA is amplified by PCR techniques and digested by specific restriction enzymes. The resulting DNA-fragments are analyzed by gel electrophoresis. Finally, the identified SNPs yield a specific TPMT genotype (figure 5a+b).

Alternatively, TPMT phenotype can be determined by measuring TPMT activity based on the *in vitro* conversion of 6-MP to 6-MMP [119]. Phenotyping may be more relevant in some cases, as there is a large variation in TPMT activity among individuals with the same genotype and it thus will give more information. Also, as mentioned before, phenotype can alter due to thiopurine therapy and the use of concomitant medication whereas genotype cannot. On the other side, sometimes phenotyping may lead to misclassification of a patient's TPMT status, for example after a blood transfusion [120]. Lately, the use of determining TPMT status, the exact moment when to perform geno- or phenotyping and the clinical implications are subject to discussion [60, 121]. Patients with one or two mutant alleles have less TPMT activity, consequently grossly elevated 6-TGN concentrations, leading to an increased risk for the development of bone marrow suppression [60]. Conversely, some wild-type patients with very high TPMT activity, so-called ultra-methylators, develop suboptimal 6-TGN concentrations by shunting 6-MP away to 6-MMP resulting in treatment failure [108].

In a retrospective study with 106 IBD patients, TPMT genotype predicted phenotype

which correlated to drug efficacy and toxicity: intermediate TPMT activity was associated with an increased risk of AZA toxicity (OR: 5.4), while high activity (>14 U/ml RBC) predicted treatment failure (OR: 0.21) [71]. In a prospective study with 67 patients with rheumatic disease, 6 patients were heterozygous for mutant TPMT alleles, of which 5 discontinued therapy within the first month of AZA treatment due to reduced total leukocyte counts [122]. In another study with 113 IBD patients, it was demonstrated that during the initial four months, lower TPMT activities correlated with low neutrophil counts. Also, patients with lower TPMT activity could be safely managed on a low (<2.0 mg/kg) AZA dose [123]. Similar results were reported in a cohort of 71 patients with CD: patients with normal TPMT status received AZA in an initial dose of 2-2.5 mg/kg/day compared to 1-1.5 mg/kg/day for patients with intermediate enzyme activity and neither developed acute leukopenia [124]. In general, a 50% starting dose, that is 1-1.5 mg/kg/day AZA or 0.75 mg/kg/day 6-MP, is advocated in patients with heterozygous TPMT alleles [125]. Some recommend an even safer initial dose reduction to 33% of the standard dose in heterozygotes [126]. It is generally recommended that homozygous mutants should not receive thiopurines at all for their IBD [125, 126]. Recently however, three case-reports demonstrated that TPMT deficiency does not preclude thiopurine therapy and hence offers a further option for homozygous mutants; these patients were safely treated with 0.16-0.29 mg/kg AZA daily, which is approximately 10% of standard dose [127].

In several other studies no or very poor correlation was found between TPMT status and thiopurine toxicity. In a study with 41 leukopenic patients with CD treated with AZA, only 27% of the patients had one or two mutant TPMT alleles [128]. Myelosuppression was more often caused by other factors, such as viral infections, drugs interfering with AZA metabolism (allopurinol and 5-aminosalicylates) or causing bone marrow suppression by their own (trimethoprim-sulfamethoxazole, captopril and metronidazole). In the majority of cases however, no obvious cause of the developed myelosuppression was apparent. The lag time to the first appearance of myelosuppression was longer in patients with wild-type alleles though. Similar results were found in another study with 56 IBD patients: a slight trend for more frequent TPMT mutations in patients with adverse reactions on AZA or 6-MP was reported, though not statistically significant [129]. Therefore, it was concluded that TPMT genotype does not predict adverse reactions due to thiopurines in this population.

Another enzyme of interest in thiopurine metabolism is ITPase. The identification of SNPs in the ITPA gene is done in a similar way as TPMT genotyping with other primers and restriction enzymes [86]. ITPase-activity can be determined by measuring the *in vitro* conversion of ITP to IMP by an HPLC method [130]. To date, in only one study with 62 IBD patients, the association between polymorphism in the ITPA gene and adverse drug reactions to AZA therapy was investigated: the C94A ITPA deficiency-associated allele significantly correlated with adverse events (OR 4.2), such as flu-like symptoms, rash and pancreatitis [85]. Besides, in the same study, no correlation was found between these adverse events and TPMT genotype.

In conclusion, whereas TPMT genotyping seems to play a role in the prediction of early drug toxicity in patients who have not been previously exposed to AZA or 6-MP, the predictive value for drug toxicity in patients established on thiopurines may be limited [60]. Studies with larger numbers of patients are needed to confirm this hypothesis. There is little experience in ITPA genotyping in IBD populations and its predictive value remains uncertain, but the first results are promising. More and larger studies are needed to confirm and clarify these provisional data.

6-Thioguanine

Yet another strategy to avoid AZA- or 6-MP-related toxicity or to enhance efficacy is the administration of 6-TG, an agent more directly leading to the formation of the active 6-TGN and until recently used only in patients suffering from leukemia. In a small pilot-study, 6-TG was given as such to 10 ultra-methylators, that is IBD patients with a preferential overproduction of 6-MMPR when given AZA or 6-MP [56]. These therapy-resistant patients continued to have suboptimal 6-TGN levels despite dose escalation of their AZA or 6-MP and 70% experienced dose related toxicity. On 6-TG treatment, 7 out of 10 patients responded or were in remission after 1-4 months. Though 6-TGN levels were 9-fold higher on 6-TG than on 6-MP, no patient experienced a recurrence of previous hepatic or hematological toxicity. 6-MMPR concentrations were undetectable at all times. The authors concluded that 6-TG is a safer and more efficacious thiopurine in this subgroup of IBD patients resistant to 6-MP therapy.

Theoretically, the administration of 6-TG also seems an attractive approach in patients with AZA- or 6-MP-intolerance because the number of possibly toxic metabolites is strongly reduced [131]. Accordingly, several clinical studies have been performed in this patient population [132-136]. In a short-term safety assessment with 32 AZA or 6-MP intolerant IBD patients it was shown that 81% of patients were able to tolerate 6-TG in the first eight weeks of treatment [132]. Comparable results were found in another study with 21 IBD patients with rechallenged allergic reactions on AZA or 6-MP: 82% were able to tolerate 6-TG [133]. In a study with 49 AZA- or 6-MP-intolerant or -resistant IBD patients, only 10% was forced to discontinue 6-TG due to the occurrence of adverse events [134]. 6-TGN concentrations were considerably higher on 6-TG compared to the concentrations measured on AZA or 6-MP therapy in all studies. Despite higher 6-TGN levels no hematological toxicity occurred.

6-Thioguanine efficacy was demonstrated in a 24-week prospective study with 37 patients with active CD: 35% achieved remission (CDAI <150) and 57% achieved a response (decrease of >70 points in CDAI) [135]. In a follow-up study, remission was maintained for one year in 88% of responders to 6-TG [136].

Initial enthusiasm tempered when nodular regenerative hyperplasia (NRH) of the liver was reported in a substantial number of 6-TG-treated IBD patients [137-139]. NRH is a serious complication also associated with AZA and 6-MP and a frequent cause of portal hypertension [140, 141].

In conclusion, important issues like 6-TG short- and long-term safety, efficacy in IBD, pharmacokinetics and the significance of higher 6-TGN levels during a longer period of time merit further exploration before 6-TG can be recommended in routine practice or be banned.

Objectives of this thesis

The objectives of the studies performed within the scope of this thesis were to determine the surplus value in reference to thiopurine efficacy or toxicity of therapeutic drug monitoring of thiopurine metabolites (part II), genotyping TPMT and ITPA (part III) or administering 6-TG as such (part IV) in inflammatory bowel disease patients.

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Chapter 2

Safety of thiopurines in the treatment of inflammatory bowel disease

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Abstract

Thiopurines have proven efficacy in inflammatory bowel disease. However, concerns regarding toxicity have limited the use of these agents as first line of medical therapy. In clinical trials, up to 15% of patients discontinued 6-mercaptopurine or its pro-drug azathioprine prematurely due to adverse events. These events may be divided in dose-independent idiosyncratic reactions and dose-related, pharmacologically explainable toxicity. Dose-independent reactions include skin rash, fever, diarrhea and pancreatitis. Most frequently observed dose-dependent adverse events are nausea, malaise and myelotoxicity. Furthermore, dose-dependent and dose-independent hepatotoxicity may occur. Recent insights obtained by therapeutic drug monitoring in patients on azathioprine or 6-mercaptopurine have led to strategies to reduce toxicity. One strategy is to detect poor metabolizers of thiopurines by establishing the activity of the key-enzyme thiopurine S-methyltransferase. However, the clinical relevance of this strategy is still a point of debate. Another strategy is to administer 6-thioguanine, which is an agent close to the effective 6-thioguaninenucleotides. In conclusion, therapeutic drug monitoring of thiopurines resulted in strategies to reduce toxicity. The value of these strategies has yet to be proven in prospective randomized trials.

Adverse events of azathioprine and 6-mercaptopurine

The thiopurines azathioprine (AZA) and 6-mercaptopurine (6-MP) have proven efficacy in patients with inflammatory bowel disease (IBD). Therefore, these agents are prescribed on a large scale in IBD patients. However, safety concerns do exist, because serious adverse events due to thiopurines may occur. In previous clinical trials, 0-15% of patients discontinued AZA or 6-MP prematurely due to adverse events [1]. Furthermore, in a retrospective study in pediatric IBD patients, cessation of therapy was needed in 18% due to side effects [1]. The side effects of thiopurines can be divided in dose-independent and pharmacologically explainable dose-dependent events [2]. Most frequently observed dose-independent events are rash, fever and arthralgia, but also pancreatitis and hepatitis may be idiosyncratic reactions. The dose-dependent toxicity of AZA and 6-MP may largely be explained by the complex metabolism of the thiopurines, which results in a number of potentially effective or toxic metabolites. Nausea and general malaise are the most frequently observed dose-dependent reactions. Hepatotoxicity may also be dose-dependent, but the most important and potentially lethal dose-dependent adverse event is myelosuppression. The reported frequency of clinically relevant leukopenia in IBD patients varies between 2-11%, depending on the definition of leukopenia and the dose of thiopurines that was prescribed [1-5]. A large survey in 739 IBD patients demonstrated leukopenia below $3.0 \times 10^9/l$ in about 4% of patients, treated with AZA in a dose of 2 mg/kg/day [3]. Another safety concern is that thiopurines have been implicated in the development of malignant lymphoma among patients with rheumatoid arthritis and in patients with a solid organ transplantation [6-8]. Contrary to these findings, the risk of developing lymphoma in AZA- or 6-MP-treated IBD patients seems not different from that in the general population [9]. However, some cases of non-Hodgkin's lymphoma have been reported in IBD patients treated with AZA or 6-MP. Recently, Lewis and colleagues concluded in a decision analysis that the benefits of thiopurines outweigh the potential risk of lymphoma in patients with moderate to severe Crohn's disease in remission [10].

Thiopurine metabolism

After administration of AZA, the pro-drug is cleaved into nitromethylimidazole and 6-MP [11]. Thereafter, 6-MP may be metabolized under influence of 3 different enzymatic pathways, as illustrated in figure 1 [12]. At first, activation to 6-thioguanine nucleotides (6-TGN) occurs by several enzymatic steps and depends critically on the enzyme hypoxanthine phosphoribosyl transferase (HPRT). Secondly, extensive inactivation of 6-MP to 6-thiouric acid occurs by xanthine oxidase. The third pathway inactivates 6-MP by the enzyme thiopurine S-methyltransferase (TPMT), which methylates 6-MP to 6-methylmercaptopurine (6-MMP). Furthermore, TPMT enables methylation

of other compounds in the metabolic pathway, which may result in additional potentially toxic or active metabolites. TPMT activity exhibits high interindividual variation due to genetic heterogeneity. The frequency distribution of TPMT activity in Caucasian populations is trimodal: homozygous wild-type alleles with high enzyme activity in about 89% of the population, heterozygous alleles with intermediate enzyme activity in about 11% and homozygous variant alleles resulting in absence of functional activity in 1 in 300 subjects [13]. Patients with low to absent TPMT activity accumulate 6-TGN, which results in an increased risk for myelosuppression.

Therapeutic drug monitoring of thiopurines

Besides TPMT analysis, therapeutic drug monitoring of AZA and 6-MP contributes to the understanding of thiopurine toxicity and efficacy in IBD. Cuffari and colleagues demonstrated an active immunosuppressive role for 6-TGN and an association of 6-MMP with hepatotoxicity in particular [14]. These findings were confirmed by Dubinsky and colleagues in pediatric IBD patients, demonstrating an optimized clinical response to 6-MP with 6-TGN levels of above 235 picomoles/ 8×10^8 red blood cells (RBC) [12]. Belaiche and colleagues reported the results of therapeutic drug monitoring in 28 Crohn's disease patients, treated with AZA or 6-MP [15]. In 6 patients with 6-TGN levels above 250 picomoles/ 8×10^8 RBC, 5 (83%) were complete responders to 6-MP, compared with 14 (63%) in 22 patients with levels below 250 picomoles/ 8×10^8 RBC. This was not significantly different, possibly due to the small numbers. More recently, Dubinsky and colleagues demonstrated increased dose-related hepatotoxicity of 6-MP, which was associated with a rise in 6-MMP ribonucleotide levels (6-MMPR) [16]. These results implicate that optimal treatment with thiopurines, concerning efficacy

Figure 1. Thiopurine metabolism. AZA, azathioprine; 6-MP, 6-mercaptopurine; 6-MMP, 6-methylmercaptapurine; 6-TUA, 6-thiouric acid; 6-MMPR, 6-methylmercaptapurine ribonucleotides; 6-TIMP, 6-thioinosine monophosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGN, 6-thioguaninenucleotides; 6-MTGN, 6-methylthioguaninenucleotides; 6-TG, 6-thioguanine; 6-MTG, 6-methylthioguanine; XO, xanthine oxidase; TPMT, thiopurine S-methyltransferase; HPRT, hypoxanthine phosphoribosyl transferase; IMPD, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase.

and safety, will be obtained in case adequate 6-TGN levels have been reached, without high levels of methylated metabolites.

Strategies to reduce toxicity

TPMT analysis

Several studies suggested TPMT pheno- or genotype assessment before thiopurine therapy [17-19]. This information can guide dosing regimes in order to reduce toxicity. In patients with intermediate TPMT activity or heterozygous TPMT genotype, 50% initial dose reduction of AZA or 6-MP minimizes the risk of leukopenia. In a recent case report in 3 IBD patients with low to absent TPMT activity (homozygous variant TPMT genotype), dose reduction of AZA to approximately 10% (0.25 mg/kg/day) of the generally advised dose was effective without toxicity [20]. On the other hand, Colombel and colleagues previously reported that TPMT activity appeared normal in 73% of Crohn's disease patients with confirmed leukopenia below $3 \times 10^9/l$ during treatment with AZA or 6-MP [21]. Furthermore, in their study, all 4 patients (10%) with low TPMT genotype, developed leukopenia within 6 weeks, which was detected with routine monitoring of blood cell counts. This means that despite knowledge of TPMT activity in a particular patient, monitoring of blood cell counts is needed after onset of AZA or 6-MP. Furthermore, the correlation of adverse events other than myelosuppression with TPMT activity remains unclear. Therefore, in clinical practice, pre-treatment TPMT assessment in addition to blood cell count monitoring after the onset of a thiopurine is not performed routinely yet.

Thiopurine metabolite monitoring

Besides confirmation of a patient's compliance, thiopurine metabolite monitoring is a powerful instrument for thiopurine therapy optimization, because of a clear correlation between 6-TGN levels and efficacy. Furthermore, methylated metabolites contribute to thiopurine toxicity. Metabolite monitoring can classify patients into 4 separate groups [16]. At first, patients with underdosing of AZA or 6-MP, which result in low 6-TGN and 6-MMPR levels. Secondly, refractory patients with absence of clinical efficacy despite high 6-TGN levels. Thirdly, patients with extremely high 6-TGN levels, resulting in increased risk of developing myelotoxicity. Finally, AZA or 6-MP intolerant patients can be identified with predominant 6-MMPR formation, resulting in low 6-TGN levels. Therefore, therapeutic drug monitoring enables rational dose adjustments in individual patients.

6-Thioguanine as down-stream metabolite

In our opinion, the most promising way to reduce dose-dependent toxicity of thiopurines is to administer a down-stream metabolite. Preferably, this compound should lead to predominant 6-TGN formation instead of methylated metabolites without the

major implications of genetic heterogeneity for the TPMT enzyme. 6-Thioguanine (6-TG), which is used in pediatric leukemia, could meet these requirements. It is converted to 6-TGN by HPRT in a single step. Although methylation of 6-TG by TPMT may occur, the clinical relevance of methylated 6-TG remains unclear [22]. At present, several small open label studies demonstrated that administration of 6-TG in a dose of 20 to 40 mg/day in IBD patients is effective with acceptable toxicity [23-25]. The kind of adverse events on 6-TG corresponds with those observed on AZA or 6-MP. Despite high 6-TGN levels, a relatively low frequency of leukopenia was observed (3 out of 79 patients, all 3 on at least 40mg/day). Obviously, 75% of IBD patients, previously intolerant to AZA or 6-MP, tolerated 6-TG well. In a recent case report, an increase of liver enzymes during 6-TG therapy was reported in one patient [26]. However, the mechanism of hepatotoxicity in this patient remained unclear. At present, no long-term data concerning safety and efficacy of 6-TG in IBD patients are available. In patients with leukemia on maintenance therapy with 6-TG, development of non-cirrhotic portal hypertension was reported [27]. However, in these patients, much higher doses of 6-TG have been used (i.e. 60-200mg/m²) together with other cytostatic agents. Controlled trials are needed to determine long-term efficacy and safety of 6-TG in IBD patients, before 6-TG may be added to the standard medical therapy in IBD. At present, 6-TG is a promising alternative in AZA or 6-MP intolerant IBD patients.

Conclusion

New insights obtained by therapeutic drug monitoring studies and pharmacogenetics has resulted in strategies to improve efficacy and to reduce toxicity of thiopurines. The most promising strategies are therapeutic drug monitoring as guidance to dose AZA or 6-MP and direct administration of the down-stream metabolite 6-thioguanine. However, the value of these strategies has yet to be proven in prospective randomized trials.

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Part II

**Therapeutic drug monitoring of
thiopurine metabolites in
inflammatory bowel disease**

Chapter 3

Pharmacokinetics of 6-mercaptopurine in patients with inflammatory bowel disease: implications for therapy

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Abstract

Proper prospective pharmacokinetic studies of 6-mercaptopurine (6-MP) in inflammatory bowel disease (IBD) patients are lacking, which has resulted in conflicting recommendations for metabolite monitoring in routine practice. The authors have evaluated 6-MP pharmacokinetics in IBD patients, including the genetic background for thiopurine S-methyltransferase (TPMT).

Red blood cell (RBC) 6-thioguaninenucleotides (6-TGN) and 6-methylmercaptopurine ribonucleotides (6-MMPR) concentrations were measured in thirty IBD patients at $t = 1, 2, 4$ and 8 weeks after starting 6-MP, 50 mg once daily. Outcome measures included mean 6-TGN and 6-MMPR concentrations ($\pm$ 95% confidence interval (CI95%)), and their associations with TPMT genotype, 6-MP dose, hematological, hepatic, pancreatic and efficacy parameters during the 8-week-period.

Steady state concentrations were reached after 4 weeks, indicating a half life of approximately 5 days for both 6-TGN and 6-MMPR, and measured 368 (CI95%: 284-452) and 2837 (CI95%: 2101-3573) picomoles/ 8×10^8 RBCs respectively. Large inter-patient variability occurred at all time-points. TPMT genotype correlated with 6-TGN concentrations (0.576, $p < 0.01$) and patients with mutant alleles had a relative risk (RR) of 12.0 (CI95%: 1.7-92.3) of developing leukopenia. A 6-MMPR/6-TGN ratio < 11 was associated with therapeutic efficacy.

Based on this pharmacokinetic analysis, therapeutic drug monitoring is essential for rational 6-MP dosing.

Introduction

6-Mercaptopurine (6-MP) is widely used in treatment of inflammatory bowel disease (IBD) and has proven to be effective for both inducing and maintaining long-term remission of Crohn's disease (CD) and ulcerative colitis (UC) [1-3]. 6-MP is more frequently used in the United States, whereas its precursor azathioprine (AZA) is the thiopurine of choice in Europe. 6-MP is transformed along competing enzymatic pathways (for review see [4]) and individual differences probably account for variations in metabolite concentrations. Thiopurine metabolism, as depicted in figure 1, ends with the formation of 6-thioguaninenucleotides (6-TGN), which are incorporated into DNA, inducing cytotoxicity and immunosuppression [4, 5].

Currently, it is much debated whether assessment of metabolite concentrations is clinically useful in predicting both drug efficacy and toxicity and conflicting results have been published: a correlation between 6-TGN concentrations and clinical efficacy was shown in pediatric patients [6], but not in a large cohort of adult patients [7, 8]. In addition, thiopurine dosing did not correlate with clinical efficacy [6, 7]. It is also uncertain which 6-MP metabolites are responsible for specific side effects. It has been suggested that high 6-TGN concentrations are associated with leukopenia, whereas elevated

Figure 1. Thiopurine metabolism. AZA, azathioprine; 6-MP, 6-mercaptopurine; 6-MMP, 6-methylmercaptopurine; 6-TUA, 6-thiouric acid; 6-MTIMP+6-MTIDP+6-MTITP, 6-methylmercaptopurine ribonucleotides (6-MMPR); 6-TIMP, 6-thioinosine monophosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP+6-TGDP+6-TGTP, 6-thioguaninenucleotides (6-TGN); 6-MTGMP, 6-methylthioguanine monophosphate; XO, xanthine oxidase; TPMT, thiopurine S-methyltransferase; HPRT, hypoxanthine phosphoribosyl transferase; IMPD, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase; MPK, monophosphate kinase; DPK, diphosphate kinase.

6-methylmercaptopurine ribonucleotides (6-MMPR) concentrations correlate with hepatotoxicity [6]. However, the precise role of 6-MMPR remains unclear, since this metabolite also has antiproliferative properties that may result in clinical efficacy [9].

These controversies in part result from a lack of prospective pharmacokinetic data in IBD patients. Besides, the clinical implications of genetic differences in 6-MP metabolism such as the much reported thiopurine S-methyltransferase (TPMT) polymorphism remain uncertain, leading to various recommendations on the use of metabolite measurements [10, 11].

The aim of this study was to collect pharmacokinetic data of thiopurines in IBD patients in a prospective study.

Materials and Methods

Patient Selection

IBD patients in which 6-MP was indicated, aged between 18 and 75 years and attending the out-patient clinics of Maasland Hospital Sittard or St Laurentius Hospital Roermond, were eligible for the study. Considered as indications for 6-MP treatment were steroid-dependency, steroid-resistance and AZA-intolerance. Exclusion criteria were: pregnancy or expected pregnancy within 6 months, inadequate contraception in women, lactation, presence of active infection, history of tuberculosis, HIV, hepatitis B or C, severe pancreatitis (necrotizing pancreatitis or pancreatitis leading to multi-organ failure), malignancy, ongoing treatment with other immunosuppressive drugs like cyclosporine, methotrexate, thalidomide or infliximab, impaired renal function (serum creatinin > 2 times normal upper limit), elevated liver function tests (> 2 times normal upper limit) and bone marrow suppression.

Study Design

The trial design was a prospective open-label multicenter study. At entry patient demographics and clinical history were collected as well as medication use during the past year. Subsequently, 6-MP (Puri-Nethol™, tablet 50 mg, Glaxo Wellcome, Zeist, the Netherlands) was prescribed as a single oral 50 mg evening dose. Concomitant medication was continued. Laboratory parameters and disease activity scores (Crohn's Disease Activity Index (CDAI) for CD and Truelove-Witts Disease Activity Index (TWDAI) for UC) were obtained at 0 (baseline), 1, 2, 4 and 8 weeks after start of medication. Blood was drawn at each visit for measurement of 6-TGN and 6-MMPR concentrations, alanine transaminase (ALT), aspartate transaminase (AST), bilirubins, amylase, lipase, leukocytes (plus differentiation), platelets and hemoglobin (Hb).

Ethical Considerations

The protocol was approved by the local medical ethics committee and in accordance with the Helsinki Declaration. Informed written consent was obtained before enrolment.

Outcome Measures

Outcome measures were mean 6-TGN and 6-MMPR concentrations $\pm$ CI95%, correlation between either steady state 6-TGN or 6-MMPR concentrations with several other parameters including dose per kg bodyweight, TPMT genotype (*1/*2/*3A/*3B/*3C), leukocyte counts, platelet counts, Hb, the occurrence of myelotoxicity (leukocyte count $< 4.0 \times 10^9/l$, platelet count $< 100 \times 10^9/l$), alanine transaminase (ALT), aspartate transaminase (AST), amylase, lipase, bilirubins, the occurrence of hepatic toxicity (ALT > 80 U/l, AST > 80 U/l, bilirubins > 40 μ mol/l), pancreatic toxicity (amylase > 220 U/l, lipase > 120 U/l (elevations > 2 times normal 8-week-period upper limit)), CDAI (CD) or TWDAI (UC) during the 8-week-period.

Analytical Procedures

TPMT genotype

TPMT genotyping of leukocyte DNA was carried out by the laboratory of the Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, based on a previously reported assay [12]. TPMT genotyping of leukocyte DNA was carried out by polymerase chain reaction (PCR) amplification in a thermocycler Gene AMP® PCR System 9700 (Perkin Elmer, Norwalk, CT, USA) followed by digestion with specific restriction enzymes. The products were separated by a 2% agarose gel containing 1x TAE with 0.5 μ g/ml ethidium bromide and read using the GeneGenius (Syngene, Cambridge, UK). The TPMT*2 allele contains a single G238C transversion mutation. The TPMT*3A allele contains two nucleotide transition mutations, being G460A and A719G. The TPMT*3B allele contains only the G460A mutation, whereas the TPMT*3C allele only has a single A719G mutation.

G238C detection: the detection of the G238C mutation was carried out using a mutant primer P2M (5'-GTATGATTTTATGCAGGTTTG-3') or a wild primer P2W (5'-GTATGATTTTATGCAGGTTTC-3') and a reverse primer P2C (5'-TAAATAGGAACCATCGGACAC-3'). 100 μ l PCR-mix was prepared using 49 μ l H₂O, 49 μ l Reddymix™ PCR Mastermix [2.5 mM MgCl₂] (Abgene, Surrey, UK), 1 μ l P2M or P2W and 1 μ l P2C. 1 μ l of worksolution (1 part of above mentioned DNA by 10 parts TE) was used as template in 10 μ l PCR-mix. The PCR reaction was carried out using the following cycles: an initial cycle of 5 minutes at 94°C followed by 30 cycles of 30 seconds at 94°C, 1 minute at 54°C, 1 minute at 72°C, and a single final extension cycle at 72°C for 5 minutes. The PCR-products were analyzed by electrophoresis under the conditions as mentioned above. A DNA fragment was amplified with P2M and P2C primers when the G238C allele was present and with P2W and P2C primers when the wild-type allele was present.

G460A detection: the detection of the G460A mutation was done using restriction fragment length polymorphism (RFLF) analysis. The primers used for amplification by PCR were G460Afor (5'-ATAACAGAGTGGGAGGCTGC-3') and G460Arev (5'-CTA-GAACCCAGAAAAAGTATAG). 100 μ l PCR-mix was prepared using 49 μ l H₂O, 49 μ l Reddymix™ PCR Mastermix, 1 μ l G460Afor and 1 μ l G460Arev. 1 μ l of worksolution (1 part of above mentioned DNA by 10 parts TE) was used as template in 10 μ l PCR-mix.

The PCR reaction was carried out in the same way as with the G238C detection. The PCR-products were digested by the endonuclease MwoI (New England Biolabs) for 3 hours at 60°C. 10 µl digestion mix consisting of 0.1 µl MwoI [5 u/µl], 2 µl MwoI-buffer and 7.9 µl H₂O was used per 10 µl PCR-products. Digested products were analyzed by electrophoresis under the conditions as mentioned above. MwoI digestion of wild-type DNA yields fragments of 267 and 98 base pairs, whereas DNA containing the G460A mutation is not digested and yields an uncleaved fragment of 365 base pairs.

A719G detection: this detection was also carried out by RFLP analysis. The primers used for this amplification by PCR were p719f (5'-GJGKJGKJHGJHG-3') and p719r (5'-GJHFGFHJGEJH-3'). 100 µl PCR-mix was prepared using 49 µl H₂O, 49 µl Reddymix™ PCR Mastermix, 1 µl p719f and 1 µl p719r. 1 µl of worksolution (1 part of above mentioned DNA by 10 parts TE) was used as template in 10 µl PCR-mix. The PCR reaction was carried out using the following cycles: an initial cycle of 5 minutes at 94°C followed by 30 cycles of 30 seconds at 94°C, 40 seconds at 55°C, 1 minute at 72°C, and a single final extension cycle at 72°C for 5 minutes. The PCR-products were digested by the endonuclease AccI (New England Biolabs) overnight by 37 °C. Per 10 µl PCR-products was used 20 µl digestion mix consisting of 0.05 µl AccI [10u/µl], 3 µl NEB4-buffer and 16.5 µl H₂O. Digested products were analyzed by electrophoresis under the conditions as mentioned above. DNA containing the A719G mutation was digested in two fragments of 201 and 62 base pairs, whereas wild-type DNA was not digested.

6-TGN and 6-MMPR concentrations

6-TGN and 6-MMPR concentrations were measured in the laboratory of the Department of Clinical Pharmacy, Maasland Hospital, Sittard, using a slightly modified assay previously reported by Lennard and colleagues [13]. Erythrocytes are a good surrogate matrix as they are easily obtained, exist in sufficient numbers and contain concentrations of 6-TGN that reflect concentrations of 6-TGN in the less accessible putative target tissue, the leukocytes [14]. All modified procedures are described.

Sample preparation

Whole blood samples were collected in coated lithium heparin tubes at least 12 hours after medication intake. The samples were centrifuged at 160 g for 10 minutes and plasma and "buffy coat" were discarded. RBCs were washed with a same volume of phosphate buffered saline (PBS) solution, mixed and centrifuged at 160 g for 10 minutes again. The supernatant was removed, RBCs were washed following the described procedure once again and centrifuged at 640 g for 10 minutes. Finally, the RBCs were resuspended in the same volume of PBS solution and erythrocyte counts were carried out with a Cell Dyn 4000 cell counter (Abbott, Hoofddorp, The Netherlands). Samples were stored at -20°C until required.

Extraction

For measurement of intracellular thionucleotides the free base was obtained by acid

hydrolysis of the nucleotide back to the purine. In brief, 200 μ l RBCs (approximately 8×10^8 RBCs) were added to a 10-ml round-bottomed extraction tube containing 500 μ l water and 300 μ l of 10 mM DL-dithiothreitol (DTT). To this was added 500 μ l 1.5 M sulphuric acid and the tubes were heated at 100°C for exactly 1 hour in a ThermoChem heating block (Marius, Germany). After cooling, 500 μ l 3.4 M sodium hydroxide was added, followed by 5 ml of 1 mM phenylmercuric chloride in dichloromethane. After shaking and centrifugation at 3500 rpm for 5 minutes, 3 ml of the organic layer was transferred to another 10-ml round-bottomed extraction tube and 200 μ l of 0.1 M hydrochloric acid was added. Again the tubes were shaken and centrifuged at 3500 rpm for 5 minutes. Finally, 10 μ l 10 mM DTT was added to 100 μ l of the acid layer and the solution was transferred to an HPLC injection vial.

HPLC procedure

For 6-thioguanine (6-TG) determination 40 μ l of the acidic extract was injected through a 717plus Autosampler (Waters Chromatography BV, Etten-leur, The Netherlands) onto a NovaPak C₁₈ column (3,9x150mm), 4 μ m particle size (Waters Chromatography BV). 6-TG was detected by a 2487 Dual λ Absorbance UV Detector (Waters Chromatography BV) set at 343 nm and the chromatographic traces were stored on a personal computer for further analysis using Millennium³² Chromatography Manager software (Waters Chromatography BV). A model 510 HPLC pump (Waters Chromatography BV) was used for solvent delivery. The mobile phase consisted of 50 mM orthophosphoric acid and 0.5 mM DTT. For 6-methylmercaptopurine (6-MMP) determination, 30 μ l of the acidic extract was processed as indicated above with the following modifications: the wavelength was set at 303 nm and the mobile phase consisted of a methanol:water mixture (4:96 v/v), containing 150 mM triethylamine and 0.5 mM DTT, adjusted to pH 3.2 with orthophosphoric acid 85%.

Calibration

Calibration curves were constructed by spiking RBCs with 6-TG (Sigma, St Louis, USA) in the range of 120-3600 picomoles/ 8×10^8 RBCs and 6-MMP (Sigma, St Louis, USA) in the range of 700-36000 picomoles/ 8×10^8 RBCs. These curves were linear with correlation coefficients of 0.992 and 0.998 for 6-TG and 6-MMP. Control samples were obtained and prepared by pooling blood samples of several patients on 6-MP treatment. The run-to-run coefficient of variation was 6.6% and 9.7% for 6-TG and 6-MMP respectively. The lower limit of quantification of the assay was determined at 30 picomoles/ 8×10^8 RBCs for 6-TG and 300 picomoles/ 8×10^8 RBCs for 6-MMP.

Statistical analysis

Normality was tested by the Kolmogorov-Smirnov test. Data are expressed as means with range or 95% confidence interval (CI95%). Pearson's correlation was used to test the relationship between the measured parameters. The χ^2 test was used to test the association between TPMT genotype and the occurrence of leukopenia. P values < 0.05

were considered significant. SPSS for Windows (version 10.0.7) software was used to perform statistics.

Results

Patients

Patient characteristics, reasons for 6-MP initiation and for study failure are shown in table 1. Thirty patients were enrolled between March 2001 and June 2002. Of the 30 patients that started 6-MP treatment, 23 patients (77%) were on 6-MP treatment for 4 weeks and 17 patients (57%) completed the 8-week-period. Steady state was reached after a period of 4 weeks. Thirteen patients failed to reach the end of the observed period and three failures were classified as non-6-MP related (table 1). Drug intolerance in 2 patients included generalized malaise in one and palpitations/headache in the other, the latter patient also demonstrating intolerance to previous AZA treatment. The other AZA-intolerant patient suffered from acute pancreatitis.

Metabolite concentrations and TPMT genotype

Metabolite concentrations were normally distributed. Mean 6-TGN and 6-MMPR concentrations are shown in table 2. Individual 6-TGN and 6-MMPR level curves are shown in figure 2 and 3 respectively. No correlation was found between steady state 6-TGN and 6-MMPR concentrations or between either 6-TGN or 6-MMPR level and dose per kg bodyweight. The TPMT genotype (wild-type versus mutant-type TPMT) showed a significant and clinically important correlation with 6-TGN concentrations (0.576, CI95%: 0.18-0.81, $p < 0.01$), but not with 6-MMPR concentrations.

Adverse events

Ten patients discontinued therapy because of adverse events (table 1). Only for the occurrence of leukopenia, a significant correlation with TPMT genotype was observed with an increase in relative risk (RR) of 12.0 (CI95%: 1.7-92.3) (table 3). Notably, 6-TGN concentrations in all 4 patients who developed leukopenia were above 300 picomoles/ 8×10^8 RBCs in week 1.

TPMT genotype was not predictive of drug intolerance, hepatotoxicity or the development of pancreatitis. 6-TGN did not show any correlation with hepatotoxicity or other adverse events. In this study, 6-MMPR concentrations did not correlate with ALT, AST, amylase, lipase, bilirubins or the occurrence of hepatotoxicity. In 1 of the 3 patients who developed pancreatitis however, the 6-MMPR level was considerably high (8224 picomoles/ 8×10^8 RBCs after 6 weeks).

Efficacy

Disease activity was assessed in all patients at baseline and after 8 weeks. Ten of the 30 patients had active disease at baseline (table 4). The mean CDAI of the 6 CD patients

Table 1. Patient characteristics

	n=30
Sex (M/F)	20/10
Age (yrs) (mean + range)	41 (19-68)
Disease (CD/UC)	19/11
Duration of disease (yrs) (mean + CI95%)	6.5 (4.1-8.9)
Disease activity at baseline	
CD remission, CDAI <150	12
active, CDAI 150-300	6
unknown	1
UC remission, TWDAI <6	5
active, TWDAI >6	4
unknown	2
Quality of Life at baseline*	62
5-ASA / no 5-ASA	28/2
5-ASA dose (mg) (median + range)	3000 (1500-4000)
Reason for 6-MP initiation	
Disease activity/steroid dependency	28
AZA-intolerance	2
6-MP dose (mg/kg) (mean + CI95%)	0.71 (0.66-0.76)
TPMT genotype	
*1/*1	20
*1/*3A	2
*1/*3C	2
*3A/*3A	1
not determined	5
Study failures	13
Non 6-MP related	3
6-MP related	10
intolerance	2 (1 AZA intolerant patient)
leukopenia	4
hepatotoxicity	1
pancreatitis	3 (1 AZA intolerant patient)

M, male; F, female; CD, Crohn's disease; UC, ulcerative colitis; CDAI, Crohn's disease activity index; TWDAI, Truelove-Witts disease activity index; 5-ASA, 5-aminosalicylic acid (-analogues); 6-MP, 6-mercaptopurine; AZA, azathioprine; TPMT, thiopurine S-methyltransferase; *) according VAS-score (0-100%).

was 229, the mean TWDAI for the 4 UC patients was 10. Three of 6 CD patients (50%) achieved clinical remission (CDAI < 150) after the study period of 8 weeks, versus 3 of 4 UC patients (75%).

Five of the 6 patients (83%) with active disease at baseline and clinical remission at week 8 had 6-TGN concentrations above the recently proposed lower therapeutic limit of 235 picomoles/8x10⁸ RBCs [6]. Three of the 4 patients (75%) with no clinical improvement had concentrations below the proposed threshold. Interestingly, the proposed therapeutic 6-MMPR/6-TGN ratio of 11 [15] also correlated with clinical efficacy: 4 of 6 IBD patients (67%) with a ratio < 11 achieved clinical remission within the study period, whereas 3 of 4 patients (75%) with a ratio > 11 failed to do so.

Figure 2. Individual 6-TGN concentration curves. The 4 patients who discontinued 6-MP therapy because of the occurrence of leukopenia are circled and their corresponding TPMT genotypes are displayed. The therapeutic lower and upper limits suggested in the literature are indicated [6]. 6-TGN, 6-thioguaninenucleotides; RBC, red blood cell; 6-MP, 6-mercaptopurine; TPMT, thiopurine S-methyltransferase.

Figure 3. Individual 6-MMPR concentration curves. The 3 patients who discontinued 6-MP therapy because of the occurrence of pancreatitis are circled, and the 1 patient with drug-induced hepatotoxicity is displayed with a square. The therapeutic upper limit suggested in the literature is indicated [6]. 6-MMPR, 6-methylmercaptopurine ribonucleotides; RBC, red blood cell; 6-MP, 6-mercaptopurine.

Table 2. 6-TGN and 6-MMPR concentrations

Week		RBC 6-TGN concentrations (in picomoles/8x10 ⁸ RBC)	RBC 6-MMPR concentrations (in picomoles/8x10 ⁸ RBC)
1	(n=30)	236 (149-323)	1691 (1316-2066)
2	(n=26)	306 (223-386)	2783 (2123-3443)
4	(n=23)	368 (284-452)	2837 (2101-3573)
8	(n=17)	355 (262-447)	2318 (1327-3308)

6-TGN, 6-thioguaninenucleotides; 6-MMPR, 6-methylmercaptapurine ribonucleotides; data are expressed as means (with 95% confidence interval).

Table 3. TPMT genotype and metabolite concentrations of all leukopenic patients

Patient	TPMT genotype	Week	RBC 6-TGN concentrations (in picomoles/8x10 ⁸ RBC)	RBC 6-MMPR concentrations (in picomoles/8x10 ⁸ RBC)
1	*3A/*1	4	628	362
2	*3A/*3A	1	1284	0
3	*1/*1	8	670	337
4	*3C/*1	2	894	2707

TPMT, thiopurine S-methyltransferase; RBCs, red blood cells; 6-TGN, 6-thioguaninenucleotides; 6-MMPR, 6-methylmercaptapurine ribonucleotides.

Discussion

Despite a large body of recent research on metabolite determination and TPMT genotyping in IBD patients treated with thiopurines, there are no uniform therapeutic guidelines and it is unclear whether such measurements should guide dosing. Recently, it has been stated that the confusion over this “correct” dosing of purine analogues was partly caused by “the lack of typical dose-ranging and pharmacokinetic studies in this patient population” [16]. We therefore prospectively performed such a study in 30 IBD patients. No distinction was made between CD and UC patients, because we did not expect a difference in thiopurine metabolism or toxicity.

We found that both 6-TGN and 6-MMPR concentrations showed large inter-patient variability, similar to results from pharmacokinetic studies in other patient populations [17, 18]. 6-TGN and 6-MMPR concentrations reached steady state after 4 weeks, suggesting a half-life of approximately 5 days. For 6-TGN concentrations, this was already demonstrated in other studies, but the half-life of 6-MMPR has not been previously reported [19]. At steady state there was a 7-fold range in 6-TGN concentrations and this is an underestimation as patients with extremely high 6-TGN concentrations discontinued 6-MP treatment after 1-2 weeks because of leukopenia. Steady state 6-MMPR concentrations reached an even larger 16-fold range.

As has been reported in children suffering from lymphoblastic leukemia, no correla-

Table 4. Disease activity and metabolite levels in patients with active disease at baseline

Active Crohn's disease (CDAI>150)		Remission after 8 wks (n=3)		6-MMPR/6-TGN ratio		Active disease after 8 wks (n=3)		6-MMPR/6-TGN ratio	
C _{ss} RBC 6-MMPR/6-TGN concentrations (picomoles/8x10 ⁶ RBCs)	Pt 1	600/180		3.3		Pt 4	2196/134		16.4
	Pt 2	2290/585		3.9		Pt 5	4520/364		12.4
	Pt 3	5193/486		10.7		Pt 6	1298/156		8.3
Active ulcerative colitis (TWDAl>6)		Remission after 8 wks (n=3)		6-MMPR/6-TGN ratio		Active disease after 8 wks (n=1)		6-MMPR/6-TGN ratio	
C _{ss} RBC 6-MMPR/6-TGN concentrations (picomoles/8x10 ⁶ RBCs)	Pt 7	5412/288		18.8		Pt 10	3740/203		18.4
	Pt 8	3166/277		11.4					
	Pt 9	2295/330		7.0					

CD, Crohn's disease; CDAI, Crohn's Disease Activity Index; C_{ss}, steady state level; UC, ulcerative colitis; TWDAl, Truelove-Witts Disease Activity Index; RBCs, red blood cells; 6-TGN, 6-thioguaninenucleotides; 6-MMPR, 6-methylmercaptopurine ribonucleotides; Pt, patient.

tion was found between 6-TGN and 6-MMPR concentrations [18]. Drug dose per kg bodyweight correlated neither with 6-TGN nor with 6-MMPR concentrations. These data do not support the use of standard doses of 1-2 mg/kg/day as is commonly advised, because this results in unpredictable 6-MP metabolite concentrations.

TPMT genotype correlated with 6-TGN concentrations, even though patients with mutant TPMT alleles who discontinued 6-MP before reaching steady state metabolite concentrations were not included in statistical analysis. It has been reported that the presence of mutant alleles of the gene encoding TPMT results in elevated 6-TGN concentrations and leukopenia [20]. In our study 5 patients with mutant alleles were identified and 3 (60%) developed leukopenia within 4 weeks of treatment. In all leukopenic patients high 6-TGN concentrations were measured and the highest 6-TGN level was measured in the patient homozygous for the TPMT*3A alleles (1284 picomoles/ 8×10^8 RBCs after 1 week). No leukopenia was observed in the other 2 patients with heterozygous alleles (steady state 6-TGN concentrations of 786 and 427 picomoles/ 8×10^8 RBCs). One of the 20 patients (5%) with wild-type alleles developed leukopenia. This patient had a steady state 6-TGN level of 670 picomoles/ 8×10^8 RBCs, which is substantially higher than the measured mean steady state 6-TGN level of 368 picomoles/ 8×10^8 RBCs. The risk of developing leukopenia was 12-fold higher in the patients with mutant alleles compared to patients with wild-type alleles. Similar results were reported in a rheumatic population treated with AZA [21]. All patients that subsequently developed leukopenia had 6-TGN concentrations above 300 picomoles/ 8×10^8 RBCs in week 1. Mean steady state concentrations would theoretically have been above 500 picomoles/ 8×10^8 RBCs in this group of patients (mean 6-TGN level week 4 / mean 6-TGN level week 1 ≈ 1.7). In fact, the mean steady state 6-TGN level in leukopenic individuals in another study was 490 picomoles/ 8×10^8 RBCs [6]. Hence, a dose reduction would be recommended if a 6-TGN level above 300 picomoles/ 8×10^8 RBCs is measured after 1 week of 6-MP treatment.

If the results of TPMT genotyping had been known prior to initiation of 6-MP treatment, at least one episode of leukopenia (in the patient homozygous for the TPMT*3A alleles) would have certainly been prevented and in the patients with heterozygous TPMT alleles, a 50% starting-dose reduction in combination with weekly TDM until steady state probably would have reduced the risk of developing leukopenia.

This study was not powered to study therapeutic efficacy. However, we found therapeutic efficacy to be associated with a 6-TGN level above the recently proposed lower therapeutic limit of 235 picomoles/ 8×10^8 RBCs. Though the described number of patients with efficacy data is small, these data are in accordance with the results of Dubinsky and colleagues [6]. In addition, a 6-MMPR/6-TGN ratio < 11 correlated with drug efficacy as was also previously suggested [15]. We must be reserved in drawing conclusions for the group of non-responders too early, as the clinical effect of 6-MP may have a delay of 3-6 months.

Steady state 6-MMPR concentrations did not correlate with any of the hepatic or pancreatic parameters. Three patients developed pancreatitis and in one the 6-MMPR con-

centration was high: although the 6-MMPR level seemed normal after 4 weeks of treatment (3308 picomoles/ 8×10^8 RBCs), concentrations rapidly increased in week 5 (7420 picomoles/ 8×10^8 RBCs) and week 6 (8224 picomoles/ 8×10^8 RBCs). In the other 2 patients steady state 6-MMPR concentrations were 1558 and 4989 picomoles/ 8×10^8 RBCs respectively. Therefore, although high 6-MMPR concentrations are sometimes detected in patients developing pancreatitis, pancreatitis often occurs in the absence of toxic 6-MMPR concentrations (>5700 picomoles/ 8×10^8 RBCs) and the role of 6-MMPR in the pathogenesis of pancreatitis is uncertain [6, 22].

In one patient (TPMT genotype $*1/*1$) the 6-MP dose was raised to 100 mg once daily because of suboptimal steady state 6-TGN concentrations. As a result, 6-MMPR concentrations increased dramatically from 3740 to 12995 picomoles/ 8×10^8 RBCs in 4 weeks, and simultaneously ALT and AST increased from 47 and 32 U/l to 139 and 61 U/l respectively, suggesting drug-induced hepatotoxicity and necessitating discontinuation of 6-MP treatment. This patient obviously had preferential shunting to 6-MMPR, resulting in an increase of the 6-MMPR/6-TGN ratio from 18.4 to 32.5 after dose escalation. Recently, Dubinsky and colleagues found that only 27% of non-responders to 6-MP treatment respond to a dose escalation [15]. In most patients dose escalation did not significantly increase 6-TGN concentration, but significant increased potentially toxic 6-MMPR concentrations. The results of that study suggested that metabolite profiles provide a biochemical explanation for 6-MP resistance and 6-MMPR related toxicity. In a second patient in our study, high 6-MMPR concentrations (7155 picomoles/ 8×10^8 RBCs after 2 weeks) were accompanied by palpitations and headache leading to discontinuation of 6-MP treatment. Both patients successfully switched from 6-MP to 6-TG 20 mg orally once daily, making a causal role of 6-MMPR in the occurrence of adverse events even more likely [23].

Conclusions

Our data indicate that future study designs should consider measurement of thiopurine metabolite concentrations at week 1 (to prevent early toxicity: 6-TGN < 300 picomoles/ 8×10^8 RBCs) and 4 (to detect suboptimal dosing or non-compliance: 6-TGN > 250 picomoles/ 8×10^8 RBCs and late toxicity: 6-TGN < 500 picomoles/ 8×10^8 RBCs) in order to validate our findings.

In conclusion, in IBD patients on 6-MP treatment, large non-dose related interindividual differences in metabolite concentrations occur. Our study clearly indicates the usefulness TPMT genotyping and especially TDM in this population. Our suggestions need to be confirmed in a prospective, randomized intervention study. TPMT genotyping before start of thiopurine treatment may prevent early myelotoxicity due to thiopurine treatment in a selection of patients. TDM will facilitate the clinician to improve 6-MP efficacy by demonstrating suboptimal dosing, and predicting risks of complications such as leukopenia and hepatitis. TDM may also be helpful in revealing poor compliance.

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Chapter 4

Some cases demonstrating the clinical usefulness of therapeutic drug monitoring in thiopurine-treated inflammatory bowel disease patients

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Abstract

The thiopurines azathioprine (AZA) and 6-mercaptopurine (6-MP) are effective drugs in steroid-dependent and -refractory inflammatory bowel disease patients. Therapeutic drug monitoring (TDM) is a new concept to improve efficacy and prevent toxic adverse events. As thiopurine metabolism is influenced by genetic polymorphisms of methylating enzymes, metabolite levels may vary considerably, enabling significant adverse effects. In present paper five patients are described to demonstrate the clinical usefulness of TDM when applying thiopurines for inflammatory bowel disease. Emphasized are patients with liver function test abnormalities and myelosuppression due to inappropriate 6-MP metabolite levels and subsequently the treatment of these events. In addition, sophisticated 6-MP metabolite level-guided therapy, including non-compliance, is demonstrated. These cases demonstrate that TDM may improve efficacy and safety of thiopurine treatment.

Introduction

The thiopurines azathioprine (AZA) and 6-mercaptopurine (6-MP) are established drugs in steroid-dependent and -refractory inflammatory bowel disease (IBD) [1,2]. New ideas in improving drug efficacy and minimizing toxicity have emerged last years. In this regard concepts have been developed including therapeutic drug monitoring (TDM) by measuring metabolites of AZA and 6-MP. After elucidating thiopurine metabolism and analytical procedures of metabolite measurement some cases are presented emphasizing the clinical usefulness of TDM in thiopurine-treated IBD patients.

AZA is rapidly converted to 6-MP, which is metabolized to the pharmacologically active 6-thioguaninenucleotides (6-TGN) and 6-methylmercaptopurine ribonucleotides (6-MMPR). 6-TGN are purine nucleotides, which are incorporated into DNA of leukocytes, inducing immunosuppression [1-4].

Historically, controlled trials demonstrated that AZA at a dose of 2.0-3.0 mg/kg/day and 6-MP at 1.5 mg/kg/day (equivalent of AZA of 3.0 mg/kg/day) are effective and generally well tolerated [1,5-8]. More recently, Cuffari and Dubinsky found in pediatric IBD patients that the clinical response only correlates with 6-thioguanine (6-TG) levels and not with other variables, such as drug dose [9,10]. These observations were the first indices of the usefulness of TDM in thiopurine-treated patients. Otherwise, authors described conflicting results in adult IBD patients [11,12]. Furthermore, it was demonstrated that red blood cell (RBC) 6-TG levels correlate with bone marrow suppression, while on the other hand 6-MMP levels correlate with hepatotoxicity [9, 13]. The lower therapeutic limit of 6-TG was established at 235 picomoles per 8×10^8 RBC, whereas 6-MMP levels above 5700 picomoles per 8×10^8 RBC seemed potentially hepatotoxic [9,10].

A drawback of AZA and 6-MP is the occurrence of side effects, leading to discontinuation of thiopurine therapy in 10-20% of patients [14-16]. Adverse events in thiopurine treatment can also be non-metabolic, like pancreatitis. The incidence of pancreatitis and hepatotoxicity by thiopurine treatment varies between 4-17 % for both independently [9,13,14].

Toxicity due to metabolites may be explained by polymorphisms of the gene encoding thiopurine S-methyltransferase (TPMT), the enzyme that methylates 6-MP to 6-MMP. Eighty-nine percent of individuals has normal to high enzyme activity. These individuals are homozygous $TPMT^{H/H}$ for the wild-type *1 allele [17]. Eleven percent has intermediate (heterozygous $TPMT^{H/L}$) and 0.3 % has low to absent enzyme activity (homozygous $TPMT^{L/L}$) [9]. $TPMT^*3A$ is the most frequently occurring variant allele ($TPMT^L$) in Caucasians [17,18]. Other mutations are *2, *3B, *3C, *3D, *4, *5, *6, *7 and *8. In case of reduced enzyme activity toxic 6-TG levels may arise on regular doses of AZA or 6-MP causing myelosuppression [19]. Alternatively, in some patients high 6-MMP levels are noted, while 6-TG levels remain subtherapeutic. Theoretically, the available 6-MP is largely transformed into 6-MMP due to preferential metabolism through TPMT, increasing the risk of hepatitis. There is no clinical evidence for this hypothesis yet. We recently performed a study with TDM and TPMT genotyping in 6-

Figure 1. Thiopurine metabolism. Note: laboratory measurements consist of a combination of several metabolites: measured 6-methylmercaptopurine (6-MMP) level, 6-MMP and 6-methylmercaptopurine ribonucleotides (6-MMPR) (=6-MTIMP+6-MTIDP+6-MTITP); measured 6-thioguanine nucleotides (6-TGN) level, 6-thioguanine (6-TG) and 6-TGN (=6-TGMP+6-TGDP+6-TGTP). AZA, azathioprine; 6-MP, 6-mercaptopurine; 6-TUA, 6-thiouric acid; 6-TIMP, 6-thioinosine monophosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTG, 6-methylthioguanine; XO, xanthine oxidase; TPMT, thiopurine S-methyltransferase; HPRT, hypoxanthine phosphoribosyl transferase; IMPD, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase; MPK, monophosphate kinase; DPK, diphosphate kinase.

MP-treated IBD patients and found no relation between TPMT and hepatitis (publication in progress).

Theoretically, 6-TG itself would be a good alternative in patients with AZA- and 6-MP-intolerance. The idea of using a metabolite at the end of the enzymatic pathway seems to be an attractive approach. Until now few short term studies in 6-TG-treated IBD patients have been published, which showed no clinically relevant toxic adverse events [20,21]. Recently, the first published long term study described improvement of disease activity in 82 percent of 21 patients, treated with 6-TG because of AZA- or 6-MP-intolerance [22]. Four patients (19%) had a harmless hypersensitivity reaction and no serious adverse events occurred. In addition, one year follow-up of 35 6-TG-treated IBD patients in our hospital did not reveal any toxicity (publication in progress). However, in thiopurine-treated leukemia and renal transplant patients, secondary tumors and portal hypertension caused by nodular regenerative hyperplasia or hepatoportal sclerosis have been described [23,24]. Concomitant immunosuppressive medication could contribute to these findings as well. More and prolonged experiences with 6-TG treatment are warranted before the widespread use of it.

Analytical procedures

Blood samples are taken at least 12 hours after medication intake and centrifuged to isolate erythrocytes. A slightly modified high-performance liquid chromatography (HPLC) assay as reported by Lennard et al. has been implemented to measure thio-purine metabolites in erythrocytes [25]. It should be emphasized that 6-TG and 6-MMP levels consist of a sum of several metabolites including 6-mercaptopurine ribonucleotides (6-MMPR) and 6-thioguanine phosphates (nucleotides or 6-TGN) as shown in figure 1. The run-to-run coefficient of variation in our laboratory is 6.6% and 9.7% for 6-TG and 6-MMP respectively. The detection limit of the assay is 30 picomoles/ 8×10^8 RBC for 6-TG and 300 picomoles/ 8×10^8 RBC for 6-MMP.

Case reports

Case 1

A 48-year-old man known with left sided ulcerative colitis (UC) for eleven years was unsuccessfully treated with betamethasone enemas, mesalazine and AZA 100 mg daily (1.0 mg/kg). After detecting zero levels of 6-MMP and 6-TG the patient admitted that he had not taken AZA for a year but had not dared to tell his gastroenterologist.

Case 2

A 50-year-old man with left sided UC for 14 months had been unsuccessfully treated with budenoside, mesalazine enemas and prednisone 30 mg daily. When starting 50 mg 6-MP daily (0.6 mg/kg), he had moderately severe disease activity according to Truelove-Witts [26].

Because of insufficient clinical improvement and subtherapeutic 6-TG levels (203 picomoles/ 8×10^8 RBC), 6-MP dose was increased to 100 mg daily (1.2 mg/kg) after 4 weeks. Three days later the patient complained about nausea and slightly increased serum transaminase levels were measured (ALAT 139 U/l (5-50) and ASAT 73 U/l (5-40)). Two weeks after dose escalation, his 6-TG level had risen to 348 picomoles/ 8×10^8 RBC and 6-MMP even to 8686 picomoles/ 8×10^8 RBC. After changing 6-MP treatment to 20 mg 6-TG (Lanvis™, GlaxoWellcome, Zeist, The Netherlands) (0.3 mg/kg), nausea disappeared and serum transaminases normalized as 6-MMP levels decreased to zero. At present, steady state 6-TG level is 884 picomoles/ 8×10^8 RBC after 8 weeks of therapy. Afterwards, TPMT genotyping revealed a wild-type or homozygous TPMT^{H/H} (*1/*1) genotype.

Case 3

A 60-year-old man with UC for eleven years treated with olsalazine 1000 mg t.i.d. experienced a flare of the disease combined with a perianal abscess. The latter was treated surgically. Thereafter, 6-MP 50 mg (0.7 mg/kg) was added to treatment. After one week

he already had 6-TG levels of 425 and 6-MMP of 482 picomoles/ 8×10^8 RBC. Two months later it was decided to decrease 6-MP dose to 25 mg (0.4 mg/kg) daily because of high 6-TG levels (628 picomoles/ 8×10^8 RBC) compared to 6-MMP (362 picomoles/ 8×10^8 RBC). On this dose steady state 6-TG levels are 417 picomoles/ 8×10^8 RBC while 6-MMP is undetectable. Disease activity is in remission. Subsequently, TPMT analysis showed a heterozygous genotype: TPMT^{L/H} (*3A/*1).

Case 4

A 32-year-old man with UC for 8 years was treated with mesalazine and prednisolone without effect. One week after starting 50 mg 6-MP daily (0.5 mg/kg) he had an extremely high 6-TG level (1284 picomoles/ 8×10^8 RBC). Because of suspected poor TPMT metabolism therapy was interrupted. He did not show up for follow-up and visited another hospital where AZA 50 mg daily was started (without knowledge of the former high 6-TG level). After several weeks he developed a severe leukopenia. Genotyping revealed a poor metabolizer: TPMT^{L/L} (*3A/*3A).

Case 5

A 23-year-old female with CD for 3 months had ongoing disease activity in spite of mesalazine and prednisolone 25 mg daily. 6-MP was started at 1.7 mg/kg without controls of metabolite levels. After 7 weeks she had fever. Laboratory analysis showed a pancytopenia: hemoglobin 4.3 mmol/l (7.5-9.9), leukocytes $1.5 \times 10^9/l$ (4.0-12.0), neutrophils 16 %, thrombocytes $13 \times 10^9/l$ (150-350). 6-MP was discontinued and she was successfully treated with a broadspectrum antibiotic. No clear focus of infection was found except her CD. Metabolite levels showed an extremely rare profile: 6-MMP 57000 and 6-TG 126 picomoles/ 8×10^8 RBC. Pancytopenia recovered spontaneously within a few weeks. Genotyping revealed a wild-type TPMT^{H/H} (*1/*1) genotype.

Discussion

TDM is an established way of improving efficacy and preventing toxicity of many drugs, like gentamicin, digoxin, antiepileptics and immunosuppressives (eg. tacrolimus and cyclosporine). The described cases emphasize the potentials of TDM in thiopurine-treated IBD patients.

Case 1 is a typical case of non-compliance, as is often seen in chronic patients in general. TDM is the only method to detect this phenomenon.

The second case shows that high 6-MMP levels may give rise to a toxic hepatitis. This patient with wild-type TPMT^{H/H} genotype has a preferential metabolism of 6-MP to 6-MMP, resulting in a failure to achieve adequate clinical benefit from 6-MP treatment even after dose increase. Recently, Dubinsky found that only 27% of non-responders to 6-MP treatment (median dose 0.9 mg/kg) respond to a dose escalation [27].

The other 73% had minimal changes in 6-TGN levels after escalation, but prominent elevations of potentially toxic 6-MMP levels. This study suggests that metabolite profiles provide a biochemical explanation for 6-MP resistance. Thereby it shows that TDM can reveal a subgroup of patients resistant to 6-MP treatment, in danger of hepatotoxicity when 6-MP dose is increased.

Case 2 also demonstrates that TDM can give the clinician a false sense of security. It is important that levels are measured early, enabling a quick intervention. In this case we received the results too late to prevent hepatotoxicity.

Besides, case 2 is an example of treatment with 6-TG, a product near the end of the metabolic pathway of thiopurines as mentioned before (figure 1). It was given in an experimental setting including intensive clinical and laboratory evaluations and TDM, like described earlier [21].

In case 3 relatively high 6-TG and low 6-MMP levels were measured. Sufficient 6-TG levels were reached at a 50% starting dose. This patient is an example of an intermediate metabolizer (heterozygous TPMT^{L/H}). It also illustrates the interindividual differences in TPMT activity and corresponds with earlier findings of Dubinsky et al. in which clinical response only correlates with 6-TG levels and not with any other variable such as drug dose [9].

The fourth case represents the one out of 300 patients with no functional TPMT activity (TPMT^{L/L}) showing extremely high 6-TG levels within just one week of 6-MP treatment. This patient had a homozygous variant allele (TPMT *3A/*3A). In this case 6-TG treatment is considered.

Case 5 is extraordinarily interesting, presenting reversible pancytopenia due to extremely high 6-MMP levels. This phenomenon has not been published previously and points out the capacity of myelotoxicity due to all 6-MP metabolites and not 6-TGN alone.

The question remains when to perform TDM. In case of preferential metabolism to 6-MMP or 6-TG rapid elevations of either level may be expected in the first week. Steady state levels of either metabolite are reached within 4 weeks. Therefore we suggest to do TDM combined with liver function tests, amylase/lipase and blood cell count at week 1 to exclude early myelotoxicity and at week 4 to detect suboptimal dosing, high 6-MMP levels or non-compliance. Furthermore TDM is recommended in case of any leukopenia or suspected hepatotoxicity. Finally, non-compliance is easily detected by TDM.

TDM is not the only way of improving thiopurine treatment efficacy and safety. TPMT genotyping before starting thiopurine treatment enables identification of patients at risk for myelosuppression [18,28]. Heterozygous patients like case 3 should have a dose reduction and intensive TDM, while homozygous poor metabolizers (TPMT^{L/L}) like case 4 are candidates for treatment with 6-TG. A significant dose reduction might be effective and safe in this case too, according to a recent report [29].

Finally, TDM may provide new data on thiopurine metabolism, such as in case 5, proving myelotoxicity due to 6-MMP.

Conclusion

TDM already is an important tool in accomplishing safe and effective drug therapy in many diseases, especially when potentially toxic drugs are employed. The present article illustrates that it is worthwhile employing TDM during thiopurine treatment because of great interindividual differences in metabolism. TDM improves efficacy of treatment and diminishes risks of serious complications such as myelosuppression and hepatitis. For this purpose thiopurine metabolite levels, blood cell count, liver function tests and amylase should be measured at week 1 and 4. TDM can also be used to reveal bad compliance or undertreatment in steady state treated patients and might help clinicians to overcome their fear for overdosing.

TPMT genotyping before starting thiopurine treatment may be complementary to TDM. The TPMT genotype predicts the chance of early neutropenia while TDM can be used to adjust 6-TG and 6-MMP levels. However, TPMT genotyping is more expensive than TDM and can only be performed in very specialized laboratories. Nevertheless, genotyping may be considered in case of neutropenia or high 6-TG levels to reveal intermediate and poor metabolizers to further adjust thiopurine treatment.

Increasing experiences of TDM in AZA and 6-MP stimulate the already growing interest in pharmacogenetics and -kinetics in general. TDM might also be of use for other medications in future.

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Part III

Pharmacogenetics of thiopurines in inflammatory bowel disease

Chapter 5

**Inosine triphosphate pyrophosphatase and
thiopurine S-methyltransferase deficiency
associated genetic polymorphisms are
related to myelosuppression in inflammatory
bowel disease patients treated with
azathioprine**

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Abstract

The use of azathioprine (AZA) in inflammatory bowel disease (IBD) patients is limited by toxicity that occurs in up to 20% treated patients. Mutations in the thiopurine S-methyltransferase (TPMT) and inosine triphosphate pyrophosphatase (ITPA) genes have been associated with the occurrence of AZA related toxicity. The aim of our study was to determine the relative contribution of ITPA and TPMT mutations to the development of toxicity induced by AZA treatment in IBD patients.

ITPA (C94A, IVS2+A21C) and TPMT (G238C, G460A, A719G) genotype were assessed in 262 IBD patients (159 females, 103 males; 67 patients with ulcerative colitis, 195 patients with Crohn's disease) treated with AZA and were correlated with the development of leukopenia and hepatotoxicity.

Leukopenia (leukocyte count $<3.0 \times 10^9/l$) was observed in 4.6% of treated patients. The frequencies of mutant ITPA C94A and TPMT alleles were significantly higher in the leukopenic population compared with patients without leukopenia (16.7% and 5.4%, respectively for ITPA C94A, and 20.8% and 4% respectively for TPMT). Moreover, the ITPA C94A and TPMT mutations predicted leukopenia: ITPA C94A odds ratio 3.504; 95%CI: 1.119-10.971 ($p=0.046$), TPMT odds ratio 6.316; 95%CI: 2.141-18.634 ($p=0.004$). Neither TPMT nor ITPA genotype predicted hepatotoxicity.

ITPA C94A and TPMT polymorphisms are associated with AZA related leukopenia in IBD patients.

Introduction

Azathioprine (AZA) is widely used for the treatment of inflammatory bowel disease (IBD) and has proven efficacy in treating active disease and maintaining remission [1-3]. However, the use of thiopurines is limited by the occurrence of adverse events leading to treatment discontinuation in up to 20% of treated patients [4-6]. Myelosuppression, manifesting itself most often as leukopenia, is potentially the most serious side effect leading to severe complications [7].

Myelosuppression is a dose-dependent event caused by significantly elevated 6-thioguaninenucleotides (6-TGN) concentrations, the active metabolites of AZA [8]. Thiopurine S-methyltransferase (TPMT) is an important enzyme in thiopurine metabolism (figure 1), as it determines the balance between two active metabolite groups: the potentially hepatotoxic 6-methylmercaptopurine ribonucleotides (6-MMPR) and the myelosuppressive 6-TGN. The gene encoding TPMT is subject to a genetic polymorphism (figure 2) that has been studied intensively. It has been demonstrated that patients with one or two mutant alleles have reduced enzyme activity, leading to elevated 6-TGN concentrations, resulting in an increased risk for developing bone marrow suppression [9-11].

However, the use of determining TPMT status, the exact moment when to perform geno- or phenotyping and the clinical implications remain subject to discussion. Although TPMT genotyping seems to play a role in the prediction of early drug toxicity in patients who have not been previously exposed to AZA, the predictive value for myelotoxicity in patients established on thiopurines may in fact be limited [11]. In a

Figure 1. Proposed thiopurine metabolism. AZA, azathioprine; 6-MP, 6-mercaptopurine; 6-MMP, 6-methylmercaptopurine; 6-TUA, 6-thiouric acid; 6-TIMP, 6-thioinosine monophosphate; 6-MMPR, 6-methylmercaptopurine ribonucleotides; 6-TIDP, 6-thioinosine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGN, 6-thioguaninenucleotides; 6-MTGN, 6-methylthioguaninenucleotides; XO, xanthine oxidase; TPMT, thiopurine S-methyl transeferase; HPRT, hypoxanthine phosphoribosyl transferase; IMPD, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase; MPK, monophosphate kinase; DPK, diphosphate kinase; ITPase, inosine triphosphate pyrophosphatase.

study with 41 leukopenic patients with Crohn’s disease (CD) treated with AZA, only 27% of the patients had one or two mutant TPMT alleles. Myelosuppression was more often caused by other factors, such as viral infections, drugs interfering with AZA metabolism like allopurinol and 5-aminosalicylates or drugs causing bone marrow suppression by their own. In the majority of cases however, the etiology of myelosuppression was unknown.

Recently, another enzyme deficiency has been associated with thiopurine toxicity. Inosine triphosphate pyrophosphatase (ITPase) catalyzes the pyrophosphohydrolysis of inosine triphosphate (ITP) to inosine monophosphate (IMP) and deficiency leads to abnormal accumulation of ITP, which is not known to be associated with any defined pathology [12]. In ITPase deficient patients treated with AZA or 6-MP though, accumulation of 6-thioinosine triphosphate (6-TITP) is suggested, resulting in AZA-intolerance. The gene encoding ITPase (ITPA) is located on chromosome 20 and 5 single nucleotide polymorphisms (SNP) have been identified so far, 3 of which (G138A, G561A, G708A) are silent and 2 (C94A, IVS2+A21C) are associated with decreased ITPase activity (figure 3). C94A is the most frequently occurring SNP. Homozygotes for the C94A missense mutation have no enzyme activity at all and IVS2+A21C homozygotes have approximately 60% of normal ITPase activity. It is estimated that approximately 6% of the population are carriers for C94A and they possess 22.5% of normal enzyme activity [12]. In a study with 62 IBD patients, the C94A ITPase deficiency-associated allele significantly correlated with adverse events, such as flu-like symptoms, rash

Figure 2. Thiopurine S-methyltransferase (TPMT) genotypes. The black and white boxes represent translated and untranslated exons respectively. The grey boxes represent translated exons on mutant alleles containing a single nucleotide polymorphism (SNP); the SNP is captioned below the relevant exon.

Figure 3. Location of the five single nucleotide polymorphisms (SNPs) on the inosine triphosphate pyrophosphatase gene (ITPA). The black and white boxes represent translated and untranslated exons respectively. G138A, G561A and G708A are silent SNPs, while C94A and IVS2+A21C are missense mutations associated with decreased ITPase activity.

and pancreatitis [13]. However, these results were contradicted by another study with 73 IBD patients [14].

We investigated the association between allelic variation of both TPMT and ITPA genes and the clinical outcome in a large group of IBD patients exposed to AZA.

Material & Methods

Patient Selection

A retrospective cross-sectional study was performed at the department of Gastroenterology and Hepatology of the Academic Medical Center, Amsterdam, the Netherlands. Medical records of all consecutive IBD patients, visiting the IBD outpatient clinic from October 1995 till September 2003, were reviewed. Patients with a positive history of AZA use for whom reliable data on AZA use and related side effects could be obtained were eligible for the study. The protocol was approved by the local medical ethics committee and in accordance with the Helsinki Declaration. Informed written consent was obtained from each participant. Information about AZA treatment duration, dose, concurrent medication and AZA related side effects have been retrieved from hospital information system and patients files.

Outcome Measures

Outcome measures were TPMT (*1, *2, *3A, *3B, *3C) and ITPA (C94A, IVS2+A21C) allele frequencies and their correlation with AZA related side effects (i.e. clear temporal relation, not explained otherwise). Myelotoxicity has been defined by criteria used by Connell [7]. Either leukopenia (white blood count $<3.0 \times 10^9/l$) or/and thrombocytopenia (platelet count $<100,000 \times 10^6/l$), resolving after treatment discontinuation or dose reduction, were considered as AZA related myelotoxicity. Hepatotoxicity was defined by serum alanine transaminase (ALT) levels greater than twice the upper normal limit (45 IU/l) and resolution after withdrawal or dose reduction of AZA.

Analytical Procedures

All patients were genotyped for ITPA C94A and IVS2+A21C, and TPMT *3A, *3B, *3C and *2. Ten ml of blood was drawn from each patient by venapuncture and white blood cells were isolated and digested with SDS/proteinase K digestion. DNA extraction was performed by means of phenol/high salt extraction and ethanol precipitation. The DNA was resuspended in TE-buffer (pH 8) and the samples were stored at 4°C.

The ITPA C94A and IVS2+A21C genotypes were assessed as described previously [13]. Briefly, each mutation specific mismatched forward and reverse primers [13] (Eurogentec s.a.) were used for amplification of DNA in Reddymix™ PCR Mastermix (Abgene, Surrey, UK). The thermocycler profile was 33 cycles of 94°C for 30s, 50°C for 30s and 72°C for 30s for ITPA C94A and 34 cycles of 94°C for 30s, 50°C for 30s and 72°C for 30s for IVS2+A21C. The amplified PCR products were digested overnight at 37°C with XmnI (New England Biolabs) in a buffer supplied by the manufacturer. The digestion products were separated by electrophoresis on 3% agarose (Eurogentec s.a.) gels containing 0.5 µl /ml ethidium bromide and viewed using the GeneGenius (SynGene, Cambridge, UK).

TPMT genotyping of G460A was carried out as described before [15]. For all DNA amplification Reddymix™ PCR Mastermix (Abgene, Surrey, UK) has been used with specific primers (Eurogentec s.a.) and after digestion of the PCR amplicon with a specific endonuclease the digestion products were separated on 3% agarose as described above. To determine G460A mutation, forward 5'-ATAACAGAGTGGGGAGGCTGC-3' and reverse 5'-CTAGAACCCAGAAAAAGTATAG-3' primers have been used, with the thermocycler profile of 30 cycles of 94°C for 30s, 50°C for 30s and 72°C for 30s. The amplified PCR product was digested by MwoI (New England Biolabs) for 2 hours by 60°C in a buffer supplied by the manufacturer. The digestion resulted in two fragments of 267 and 98 bp in a wild-type while in a G460A mutation the PCR product remained undigested of 365 bp. To determine G238C variant the DNA amplification has been performed using mutant and wild-type forward primers with a common reverse primer separately for each sample as described previously [15]. The sequences of particular primers were as follows: mutant primer 5'-GTATGATTTTATGCAGGTTTC-3', wild-type primer 5'-GTATGATTTTATGCAGGTTTC-3' and reverse primer 5'-TAAATAG-GAACCATCGGACAC-3'. The thermocycler profile was 30 cycles of 94°C for 30s, 50°C for 30s and 72°C for 30s. G238C variant has been determined according to the presence or absence of 256 bp PCR product.

TPMT genotype A719G was assessed by PCR with further digestion and analysis of restriction fragment length polymorphisms. For the amplification, forward 5'-TAACAT-GTTACTCTTTCTTGTTTCAG-3' and reverse 5'-GTCAGTGTGATTTTATTTTATCTA TGTC-3' primers were used with the thermocycler profile of 30 cycles of 94°C for 30s, 50°C for 30s and 72°C for 30s. The PCR amplicon was digested by AccI (New England Biolabs) overnight by 37°C in a buffer delivered by the manufacturer. The A719G mutation creates a digestion site in original wild type fragment of 171 bp, which leads to restriction fragments of 121 bp and 50 bp.

TPMT genotypes, TPMT *3A, *3B, *3C and *2, have been determined for each patient on the basis of the three mutations studied. Since the mutations G460A and A719G are only with few exceptions combined on the same allele, the genotype TPMT *3A was then assumed for the person carrying these two mutations. Isolated G460A, A719G and G238C have been defined as TPMT *3B, *3C and *2, respectively.

Statistical Analysis

Differences in allele frequencies between respective groups were tested by Fishers exact test. Data are presented as allele frequencies and percentages of allele carriers in patients groups. Odds ratios (OR) and 95% confidence intervals (CI95%) were calculated from contingency tables. An appropriate modification of the test was performed when the observed cell count in the contingency table was 0. P-values <0.05 were considered to be statistically significant. Software program SPSS Pro for Windows (version 11.5) has been used for statistical analysis.

Results

Patients

Two hundred and sixty two IBD patients were included in this study. The mean AZA dose received in the whole patient population was 132 mg (range 50-250 mg), with a mean duration of AZA exposure of 35 months (range 1-143 months). Patient characteristics are shown in table 1.

Primary Outcomes

ITPA and TPMT allele frequencies

Allelic variants of ITPA and TPMT genes and their frequencies are shown in table 2. Homozygosity for ITPA C94A allele and IVS2+A21C alleles has been found in 1 and 4 patients, respectively. Five patients were compound heterozygotes for ITPA C94A allele and IVS2+A21C. Only TPMT *3A and *3C genotypes were identified among all TPMT deficiency associated alleles studied, with one patient being homozygote for TPMT *3A.

Azathioprine related toxic side effects

Bone marrow toxicity was observed in 12 patients (4.6%): 7 patients experienced leukopenia, 5 patients had combined leukopenia and thrombocytopenia. Leukopenia occurred after an average exposure time of 7.1 months (range 1-16). Treatment was discontinued in 6 patients due to myelotoxicity; AZA dosage was decreased in 4 patients and 2 patients experienced stable leukopenia with leukocytes counts between 2.5 and $3.0 \times 10^9/l$ during the whole treatment period with a clear temporal relationship with the start of the treatment and no other possible cause of leukopenia.

Table 1. Demographic characteristics of the patient population

		n=262
Sex (M/F)		103/159
Age (yrs) (mean + range)		39 (17-87)
Disease (CD/UC)		195/67
Duration of disease (yrs) (mean + CI95%)		11 (1-38)
Disease localization		
CD	large intestine	40 (20.5%)
	small intestine	47 (24.1%)
	combination large and small intestine involvement	107 (54.9%)
	upper GI tract	1 (0.5%)
UC	distal colitis	35 (52.2%)
	pancolitis	32 (47.8%)
Fistulizing/Non-fistulizing disease		87/108
Duration of azathioprine use in months (median + range)		35 (1-143)
Daily azathioprine dose in mg (mean + range)		132 (50-250)
Comedication during azathioprine exposure		
	5-ASA	145 (55%)
	corticosteroids	207 (79%)
	anti-TNF	34 (13%)

M, male; F, female; CD, Crohn's disease; UC, ulcerative colitis; GI, gastrointestinal; 5-ASA, 5-aminosalicylic acid (-analogues); anti-TNF, anti-tumor necrosis factor.

Table 2. Allelic variants of ITPA and TPMT gene and their frequencies among 262 IBD patients (n=524 alleles)

Allelic variants		n	%	Homozygotes/Heterozygotes
TPMT	*1	499	95.2	238/NA
	*2	0	0	0
	*3A	19	3.6	1/17
	*3B	0	0	0
	*3C	6	1.1	0/6
ITPA	ITPA C94A	31	5.9	1/29
	ITPA IVS2+A21C	63	12.0	4/55

TPMT, thiopurine S-methyltransferase; ITPA inosine triphosphate pyrophosphatase allele.

Hepatotoxicity occurred in 11 patients (4.2%) after a mean AZA exposure period of 6.1 months (range 1-19). Medication withdrawal due to hepatotoxicity was necessary in 5 patients and dose adjustment was performed in 6 patients.

Correlation of ITPA and TPMT polymorphism with azathioprine related toxic side effects

Leukopenia

A significantly higher frequency of the ITPA C94A allele was found among leukopenic patients compared to patients without leukopenia: 16.7% (4 patients) and 5.4% (26 patients), respectively (table 3). ITPA C94A allele was associated with AZA related leukopenia (OR=3.504; CI95% 1.119-10.971, p=0.046, figure 4). The only homozygote for ITPA

C94A found in the studied population did not develop leukopenia. No significant difference in allele frequency has been observed for IVS2+A21C allele in leukopenia and non-leukopenia groups, 4.2% (1 patient) and 12.4% (58 patients), respectively (figure 4). Among all included patients, four compound heterozygotes for studied ITPA deficiency associated allelic variants were found. A higher percentage of compound heterozygotes was found in leukopenic group (8.3%, 1 patient) compared with non-leukopenic patients (1.4%, 3 patients), however, this difference was not significant.

In the studied TPMT mutant alleles frequencies, significant differences were found between leukopenic and non-leukopenic group. Among leukopenic patients, 20.8% (4 patients) were carriers of mutant alleles vs. 4% (20 patients) in non-leukopenic group (table 3, figure 4). The TPMT deficiency associated alleles predicted leukopenia (OR=6.316; CI95%: 2.141-18.634, $p=0.004$).

Compound heterozygosity for studied ITPA and TPMT mutant alleles was found in 9 patients without leukopenia (3.6%) and in one leukopenic patient (8.3%, $p=0.38$). No association of compound ITPA and TPMT heterozygosity with leukopenia was observed (OR=2.434; CI95%: 0.283-20.952, $p=0.38$).

In order to assess other possible factors interfering with occurrence of leukopenia we studied the differences in AZA doses and concurrent medication between leukopenia and non-leukopenia groups. For both ITPA C94A and TPMT polymorphisms, total daily AZA dose in the wild-type allele group was higher than in the mutant group, however, the difference was not significant. This represented an average daily AZA dose of 134 mg in the wild-type group vs. 112 mg in the mutant group for ITPA C94A and 140 mg in the wild-type group vs. 100 mg in the mutant group for all TPMT polymor-

Figure 4. Percentages of ITPA and TPMT (*3A or *3C) mutant alleles carriers among AZA users with respect to the development of leukopenia. * $p=0.036$, OR=4.308; CI95%: 1.213-15.294; ** $p=0.016$, OR=5.75; CI95%: 1.592-20.769. ITPA, inosine triphosphate pyrophosphatase; TPMT, thiopurine S-methyltransferase; AZA, azathioprine.

phisms. No differences in the use of concurrent medication, i.e. 5-ASA, corticosteroids and infliximab, have been observed between the two groups. None of leukopenia patients was treated with allopurinol at the moment of leukopenia onset.

By a low enzymatic activity, as observed among homozygotes mutants for the studied TPMT and ITPA alleles, a quick onset of leukopenia would be expected. The only patient homozygote for TPMT (TPMT *3A) in our study has indeed experienced severe leukopenia during the first 2 weeks of the treatment, necessitating medication withdrawal. However, no difference in the time to leukopenia onset between ITPA and TPMT heterozygotes could be detected.

Hepatotoxicity

No significant difference between the group with and without AZA related hepatotoxicity was observed in allele frequencies for both ITPA C94A (0 patients and 6.2% - 30 patients, respectively, $p=0.633$) and IVS2+A21C alleles (4.5% - one patient and 12.4% - 58 patients, respectively, $p=0.499$). Neither ITPA C94A (OR 0.33; CI95%: 0.02-5.6, $p=0.633$), nor IVS2+A21C (OR 0.338; CI95%: 0.045-2.557, $p=0.499$) alleles were associated with hepatotoxicity.

In addition, no difference has been observed in TPMT mutant allele frequencies among patients with AZA related hepatotoxicity (4.6%, 22 patients) compared with patients without (9.1%, 2 patients) and no association of TPMT mutant allelic variants with hepatotoxicity was found (OR 2.083; CI95%: 0.459-9.452, $p=0.283$).

Neither compound heterozygosity for ITPA C94A and IVS2+A21C nor for ITPA and TPMT have been associated with AZA related hepatotoxicity (OR 2.39; CI95%: 0.12-47.13, $p=1.000$ and OR 1.00; CI95%: 0.055-18.14, $p=1.000$).

Discussion

We studied the allelic frequencies of earlier reported, and clinically important polymorphisms of TPMT and ITPA, and their association with toxicity in IBD patients treat-

Table 3. Carriage of ITPA and TPMT mutant alleles in AZA using IBD patients population with and without side effect

	ITPA C94A	ITPA IVS2+A21C	TPMT *3A and TPMT *3C
Leukopenia	4/12 (33%)	1/12 (8%)	4/12 (33%)
No leukopenia	26/250 (10%)	58/250 (23%)	20/250 (8%)
	$p=0.036$	$p=0.309$	$p=0.016$
Hepatotoxicity	0/11 (0%)	1/11 (9%)	2/11 (18%)
No hepatotoxicity	30/251 (12%)	58/251 (23%)	22/251 (9%)
	$p=0.621$	$p=0.465$	$p=0.266$

TPMT, thiopurine S-methyltransferase; ITPA inosine triphosphate pyrophosphatase allele; AZA, azathioprine; IBD, inflammatory bowel disease.

ed with AZA maintenance therapy. In this largest cohort studied so far, we found that patients carrying TPMT (G238C, G460A, A719G) and ITPA (C94A) allele mutations are at increased risk of developing leukopenia (TPMT odds ratio 6.316; CI95%: 2.141-18.634 ($p=0.004$); ITPA C94A odds ratio 3.504; CI95%: 1.119-10.971 ($p=0.046$)). In addition, we did not detect any association between either TPMT or ITPA genotype and hepatotoxicity.

Until now, the exact risk of developing leukopenia during AZA in IBD patients was not clear since several contradictory studies have been published resulting in a lively debate in the IBD community whether or not to use TPMT enzyme activity or genotyping prior to AZA administration.

It has been demonstrated indisputably that homozygous TPMT mutants, like the one patient in our cohort, will experience early and severe myelosuppression when treated with standard AZA dosages. TPMT geno- or phenotyping prior to initiation is the only way of identifying these patients at high risk [11, 16, 17]. For heterozygous TPMT mutants however, although an increased risk of developing bone marrow suppression in a subsequent stage exists, the correlation with TPMT geno- or phenotype is less pronounced [9, 16]. TPMT heterozygosity is just one of many risk factors such as concurrent medication (drugs interfering with AZA metabolism or causing bone marrow suppression by their own) or intercurrent viral infections and will not necessarily lead to myelosuppression [16, 17]. In addition, the issue of drug toxicity was further complicated with the introduction of ITPase deficiency as a risk factor for AZA toxicity in IBD. ITPase deficiency is a clinically benign condition characterized by abnormal accumulation of ITP in erythrocytes. Complete ITPase deficiency is associated with a homozygous missense C94A mutation that encodes a 32Pro>Thr exchange [12]. The speculations that 6-thio-IMP, an intermediate metabolite of AZA metabolism, might be further phosphorylated to its di- and triphosphate derivatives has lead to the hypothesis of possible accumulation of 6-thio-ITP in ITPase deficient AZA users. Although a study in 62 IBD patients observed an increase in adverse events in patients carrying the C94A mutation, a risk for leukopenia was not detected [13]. A recent letter investigating the ITPA C94A frequency in 41 patients could also not find an increased risk for the development of leukopenia [18]. The possible explanations for the discrepancies between these studies and our observations are the differences in study design and cohort size. In our study, the majority of leukopenic ITPA C94A mutant allele carriers experienced late onset of AZA related leukopenia. In the two referred studies, the design of a non cross-sectional, selective inclusion of leukopenic patients might have lead to a higher proportion of early leukopenic AZA users. This, together with a small cohort size may be the reason for a lack of association between ITPA C94A polymorphism and leukopenia as found in our study.

By a casual association of enzyme deficiency and related side effects due to metabolite accumulation one may expect a dose related effect. However, the only homozygote patient for ITPA C94A we found did not develop leukopenia by the achieved 13 months of treatment at the moment of the study closure. Therefore, we think that ITPA geno-

typing is probably not of great clinical importance and periodic lab monitoring will be sufficient to detect ITPase deficiency associated leukopenia.

In conclusion, we have demonstrated that ITPA C94A and TPMT polymorphisms are associated with AZA related leukopenia in IBD patients. However, in terms of consequences for the clinical practice, the only up-to-date known serious and preventable AZA related adverse event is leukopenia resulting from low TPMT enzymatic activity in homozygote mutants. Based upon our findings we think that TPMT genotyping or enzyme activity assessment is warranted prior to installation of AZA maintenance therapy in IBD. Although carriers of the ITPA C94A mutation also have an increased risk, current monitoring guidelines probably suffice for the adequate detection of drug-related toxicity.

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Part IV

6-Thioguanine in inflammatory bowel disease

Chapter 6

Pharmacokinetics of 6-thioguanine in patients with inflammatory bowel disease

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Abstract

6-Thioguanine (6-TG) seems to be an attractive alternative in both AZA- and 6-MP-intolerant and -resistant IBD populations. However, little is known of 6-TG pharmacokinetics in IBD patients, metabolite levels and their correlation with drug efficacy and toxicity. This study reports 6-TG pharmacokinetics in a population of IBD patients and the predictive value of metabolite concentrations.

Red blood cell (RBC) 6-thioguaninenucleotides (6-TGN) concentrations were measured in twenty-eight IBD patients at $t = 1, 2, 4$ and 8 weeks after starting 6-TG, 20 mg once daily. Outcome measures included mean 6-TGN concentrations ($\pm 95\%$ confidence interval (CI95%)), and their associations with TPMT genotype, 6-TG dose, hematological, hepatic-, pancreatic- and efficacy parameters during the 8-week-period. Steady state 6-TGN concentrations were reached after 4 weeks, indicating a half life of approximately 5 days, and measured 856 (CI95%: 715-997) picomoles/ 8×10^8 RBCs. Large inter-patient variability occurred at all time-points. No correlation was found between steady state 6-TGN concentrations and drug dose per kg bodyweight. No significant differences in 6-TGN concentrations were found between patients with adverse events versus patients without any event. Also, mean 6-TGN concentrations did not differ in patients with active disease versus patients in remission.

In IBD patients on 6-TG treatment, large interindividual differences in metabolite concentrations occur. In our population, we could not demonstrate a clear relationship between 6-TGN concentrations on one hand and toxicity and efficacy on the other like in AZA- and 6-MP-treated patients.

Introduction

6-Mercaptopurine (6-MP) and its prodrug azathioprine (AZA) have proven efficacy in the treatment of inflammatory bowel disease (IBD) [1-3]. Thiopurines themselves are inactive and require intracellular metabolism (figure 1) to the active 6-thioguanine-nucleotides (6-TGN), which are incorporated into DNA causing immunosuppression [4, 5]. Also, 6-thioguaninetriphosphate (6-TGTP), one of the 6-TGN, is considered to contribute to the immunosuppressive effects due to inhibition of Rac1 inducing apoptosis [6]. However, these immunosuppressives are ineffective in one third of patients [7] and 10-20% of AZA- or 6-MP-treated patients is forced to discontinue drug therapy due to the occurrence of adverse events [8-10]. Administration of 6-thioguanine (6-TG), an agent much closer to effective 6-TGN, seems to be an attractive alternative in both AZA- and 6-MP-intolerant [11, 12] and resistant populations [13, 14]. Recently discovered disturbing high frequencies of nodular regenerative hyperplasia (NRH) in the liver in patients treated with 6-thioguanine tempered initial enthusiasm [15].

In patients treated with 6-MP it is shown that red blood cell (RBC) 6-TGN levels correlate with both drug efficacy and myelotoxicity and a therapeutic window of 250-500 picomoles/ 8×10^8 RBC was proposed [16, 17]. However, in patients treated with 6-TG much higher metabolite levels develop without any sign of myelotoxicity [12]. In fact, very little is known of 6-TG pharmacokinetics in IBD patients, metabolite levels and

Figure 1. Thiopurine metabolism. AZA, azathioprine; 6-MP, 6-mercaptopurine; 6-MMP, 6-methyl-mercaptopurine; 6-TUA, 6-thiouric acid; 6-TIMP, 6-thioinosine monophosphate; 6-MTIMP, 6-methylthioinosine monophosphate; 6-TIDP, 6-thioinosine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-TGN; 6-thioguanine nucleotides; 6-TG, 6-thioguanine; 6-MTG, 6-methylthioguanine; XO, xanthine oxidase; TPMT, thiopurine S-methyltransferase; HPRT, hypoxanthine phosphoribosyl transferase; IMPD, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase; MPK, monophosphate kinase; DPK, diphosphate kinase; ITPase, inosine triphosphate pyrophosphatase.

their correlation with drug efficacy and toxicity. We therefore performed a prospective pharmacokinetic study of 6-TG in a population of IBD patients.

Material & Methods

Patient Selection

IBD patients in which 6-TG was indicated, aged between 18 and 75 years and attending the out-patient clinics of Maasland Hospital, Sittard or St Laurentius Hospital, Roermond, were eligible for the study. Considered as indications for 6-TG treatment were steroid-dependency, steroid-resistance and AZA- or 6-MP-intolerance (both hypersensitivity reactions and dose-dependent events). Exclusion criteria were: pregnancy or expected pregnancy within 6 months, inadequate contraception in women, lactation, presence of active infection, history of tuberculosis, HIV, hepatitis B or C, severe pancreatitis (necrotizing pancreatitis or pancreatitis leading to multi organ failure), malignancy, ongoing treatment with other immunosuppressive drugs like cyclosporine, methotrexate, thalidomide or infliximab, impaired renal function (serum creatinin > 2 times normal upper limit), elevated liver function tests (> 2 times normal upper limit) and bone marrow suppression.

Study Design

The trial design was a prospective open-label multicenter study. At entry patient demographics and clinical history were collected as well as medication use during the past year. Subsequently, 6-TG (Lanvis™, tablet 40 mg, GlaxoWellcome, Zeist, the Netherlands) was prescribed as a single oral 20 mg evening dose. Therefore, the commercial available 40 mg tablets were divided into half by a tablet-splitter in the production-unit of the Department of Clinical Pharmacy, Maasland Hospital, Sittard. Concomitant medication was continued: aminosalicylates were allowed as stable comedication throughout the study and steroid dose could be tapered according to clinical response. Laboratory parameters, disease activity scores (Crohn's Disease Activity Index (CDAI) for CD and Truelove-Witts Disease Activity Index (TWDAI) for UC) and quality of life (according VAS-score) were obtained at 0 (baseline), 1, 2, 4 and 8 weeks after start of medication. Blood was drawn each visit for measurement of 6-TGN concentrations, alanine transaminase (ALT), aspartate transaminase (AST), bilirubins, amylase, lipase, leukocytes (plus differentiation), platelets and hemoglobin (Hb). Additionally, in 3 randomly chosen patients with 6-TGN concentrations at steady state blood was drawn for the measurement of 6-TGN concentrations during a twenty-four hours' period at 0, 2, 4, 8, 12 and 24 hours after 6-TG intake to study concentration fluctuations during the day.

Ethical Considerations

The protocol was approved by the local medical ethics committee and in accordance with the Helsinki Declaration. Informed written consent was obtained before enrolment.

Outcome Measures

Outcome measures were individual 24-hours- and 8-week-curves, mean 6-TGN concentrations $\pm$ CI95%, correlation between steady state 6-TGN concentrations with several other parameters including dose per kg bodyweight, TPMT genotype (TPMT*1/TPMT*2/TPMT*3A/TPMT*3B/TPMT*3C), the occurrence of myelotoxicity (leukocyte count $< 4.0 \times 10^9$ /l, platelet count $< 100 \times 10^9$ /l), the occurrence of hepatic toxicity (ALT > 80 U/l, AST > 80 U/l, bilirubins > 40 μ mol/l), pancreatic toxicity (amylase > 220 U/l, lipase > 120 U/l (elevations > 2 times normal upper limit)), CDAI (CD) or TWDAI (UC) during the 8-week-period. The relationship of adverse events with the use of 6-TG was established by the following method: unrelated, no temporal relation and other etiology likely; possibly related, potential temporal relation and other etiologies possible; probably related, potential temporal relation and other etiologies unlikely; related, clear temporal relation not otherwise explained.

Analytical Procedures

6-TGN concentrations were measured in the laboratory of the Department of Clinical Pharmacy, Maasland Hospital, Sittard, using previously published assay [17]. Erythrocytes are a good surrogate matrix as they are easily obtained, exist in sufficient numbers and contain concentrations of 6-TGN that reflect concentrations of 6-TGN in the less accessible putative target-tissue, the leukocytes [18]. In brief, the thiopurines are separated by reversed-phase HPLC and quantified using UV-detection. For measurement of intracellular thionucleotides the free base is obtained by acid hydrolysis of the nucleotide back to the purine. The resulting purines are extracted from the biological matrix by forming a phenylmercury adduct into dichloromethane. During back-extraction with hydrochloric acid the adduct is split and the free thiopurine liberated into the acid layer once again. The run-to-run coefficient of variation was 6.6%. The lower limit of quantification of the assay was determined at 30 picomoles/ 8×10^8 RBCs. TPMT genotyping of leukocyte DNA was carried out by the laboratory of the Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, based on a previously reported assay [19].

Statistical Analysis

Normality was tested by the Kolmogorov-Smirnov test. Data are expressed as means with range or 95% confidence interval (CI95%). Pearson's correlation was used to test the relationship between the measured parameters. P values < 0.05 were considered significant. SPSS for Windows (version 10.0.7) software was used to perform statistics.

Results

Patients

Patient characteristics and reasons for 6-TG initiation are shown in table 1. Twenty-eight patients were enrolled between May 2001 and November 2003. Of the 28 patients, 27 patients (96%) were on 6-TG treatment for 4 weeks and 24 patients (86%) completed the 8-week-period. Steady state was reached after a period of 4 weeks. Four patients failed to reach the end of the observed period because of the occurrence of adverse events.

Metabolite concentrations

Metabolite concentrations were normally distributed. Mean 6-TGN concentrations are shown in table 2. Individual 8-week and 24-hours 6-TGN curves are shown in figure 2

Table 1. Patient characteristics

	n=28
Sex (M/F)	6/22
Age (yrs) (mean + range)	38 (19-70)
Disease (CD/UC)	16/12
Location of disease	
CD small bowel	5
large bowel	6
combination	5
UC distal colitis	9
pancolitis	3
Duration of disease (yrs) (mean + CI95%)	10.0 (7.4-12.6)
Disease activity at baseline	
CD remission, CDAI <150	5
active, CDAI 150-300	11
UC remission, TWDAI <6	7
active, TWDAI >6	5
Quality of Life at baseline*	56
5-ASA / no 5-ASA	23/5
5-ASA dose (mg) (median + range)	3000 (1500-3200)
Reason for 6-TG initiation	
Disease activity/steroid dependency	1
AZA-intolerance	9
6-MP-intolerance	8
AZA- and 6-MP-intolerance	8
Other	2
6-TG dose (mg/kg) (mean + CI95%)	0.32 (0.29-0.34)
TPMT genotype	
*1/*1	17
not determined	11

M, male; F, female; CD, Crohn's disease; UC, ulcerative colitis; CDAI, Crohn's disease activity index; TWDAI, Truelove-Witts disease activity index; 5-ASA, 5-aminosalicylic acid (-analogues); 6-TG, 6-thioguanine; AZA, azathioprine; 6-MP, 6-mercaptopurine; TPMT, thiopurine S-methyltransferase; *) according VAS-score (0-100%).

Table 2. 6-TGN concentrations

Week		RBC 6-TGN concentrations (in picomoles/ 8×10^8 RBC)	
1	(n=27)	481	(35-1254)
2	(n=25)	678	(123-1763)
4	(n=27)	856	(192-2112)
8	(n=24)	848	(284-1888)

6-TGN, 6-thioguaninenucleotides; data are expressed as means (with 95% confidence interval).

and 3 respectively. Seven patients had measurable 6-TGN concentrations at inclusion because AZA or 6-MP therapy was discontinued only recently. 6-TGN concentrations reached steady state concentrations after 4 weeks in all but one patient with questionable compliance. No correlation was found between steady state 6-TGN concentrations and dose per kg bodyweight, as shown in figure 4.

TPMT genotype

TPMT genotype was determined in 17 of 28 patients (61%) and all genotyped subjects possessed wild-type alleles (table 1). In 2 of the 4 patients intolerant to 6-TG TPMT genotype could not be assessed because of loss to follow-up. In the other 2 patients a wild-type TPMT genotype has been proven. The patient with the clearly highest 6-TGN concentrations possessed wild-type alleles.

Figure 2. Individual 6-TGN concentrations curves (8 weeks). 6-TGN, 6-thioguaninenucleotides; RBC, red blood cell.

Figure 3. Individual steady state 6-TGN concentrations curves (24 hours). 6-TGN, 6-thioguaninenucleotides; RBC, red blood cell. The depicted standard deviations express the margin of error of our assay for 6-TGN measurement.

Figure 4. Correlation between 6-TGN concentrations and dose in mg 6-TG per kg bodyweight. 6-TGN, 6-thioguaninenucleotides; 6-TG, 6-thioguanine; RBC, red blood cell.

Adverse events

Four patients discontinued therapy because of adverse events. Adverse events leading to discontinuation of 6-TG treatment are summarized in table 3. In these patients, 6-TGN concentrations varied considerably. No myelotoxicity or hepatotoxicity occurred in the observed period. In one patient amylase (158 U/l) and lipase (273 U/l) levels temporarily increased without any symptoms of a clinically present pancreatitis.

Table 3. Adverse events leading to discontinuation of 6-thioguanine treatment

No	Dose (in mg/kg)	Last 6-TGN level (picomoles/8x10 ⁶ RBC)	Time to AE (in days)	Character of AE	Relationship AE/6-TG
1	0.26	552	24	nausea*, diarrhea*, stomach cramps*	probably related
2	0.29	1013	42	total malaise*	possibly related
3	0.20	229	7	fever*, erythema nodosum*, arthralgia*	related
4	0.34	–	7	nausea*, stomach cramps, headache*	probably related

6-TGN, 6-thioguaninenucleotides; AE, adverse events; *) same AE as previously shown on AZA or 6-MP

Table 4. Disease activity and metabolite levels in 16 patients with active disease at baseline

Active Crohn's disease (CDAI>150)	Remission after 8 wks (n=5)		Active disease after 8 wks (n=6)	
C _s RBC 6-TGN concentrations (picomoles/8x10 ⁶ RBCs)	Pt 1	1013	Pt 6	1269
	Pt 2	819	Pt 7	1060
	Pt 3	996	Pt 8	1009
	Pt 4	1001	Pt 9	411
	Pt 5	753	Pt 10	696
			Pt 11	1155
Active ulcerative colitis (TWDAl>6)	Remission after 8 wks (n=3)		Active disease after 8 wks (n=2)	
C _s RBC 6-TGN concentrations (picomoles/8x10 ⁶ RBCs)	Pt 12	531	Pt 14	863
	Pt 13	819	Pt 15	423
			Pt 16	483

CD, Crohn's disease; CDAI, Crohn's Disease Activity Index; Css, steady state level; UC, ulcerative colitis; TWDAl, Truelove-Witts Disease Activity Index; RBCs, red blood cells; 6-TGN, 6-thioguaninenucleotides; Pt, patient.

Efficacy

Disease activity was assessed in all patients at baseline and after 8 weeks. Sixteen of the 28 patients had active disease at baseline (table 4). The mean CDAI of the 11 CD patients was 224 and the TWDAI of the 5 UC patients was 10. Five of the 11 (45%) CD patients achieved clinical remission (CDAI < 150) after the study period of 8 weeks versus 2 of the 5 (40%) UC patients. In the CD patients, the mean 6-TGN concentration in patients with active disease was 933 picomoles/ 8×10^8 RBCs (CI95%: 677-1190) compared to 916 picomoles/ 8×10^8 RBCs (CI95%: 810-1023) in patients that achieved clinical remission. In the UC patients both means were 590 (CI95%: 238-942) picomoles/ 8×10^8 RBCs and 675 (CI95%: 393-957) picomoles/ 8×10^8 RBCs for active disease and remission respectively. No significant differences in mean 6-TGN concentrations were found.

Discussion

6-Thioguanine has been available for decades for the treatment of patients suffering from leukemia, but its use was virtually abandoned by the arrival of other more effective regimens. Recently, the interest in 6-TG has been renewed as it may be an alternative for AZA- or 6-MP-intolerant or -resistant IBD patients. It is much debated whether the measurement of 6-TG derived 6-TGN, the main metabolites, should guide dosing [11, 12]. That is, therapeutic drug monitoring is suggested in AZA- or 6-MP-treated patients [16, 17]. Similar pharmacokinetic studies with 6-TG derived 6-TGN are scarce [20] and we therefore performed such a study in 28 IBD patients. No distinction was made between CD and UC patients because we did not expect a difference in thio-purine metabolism.

The absorption of oral 6-TG is incomplete and variable, resulting in a bioavailability of 14-46% [21, 22]. Plasma 6-TG concentrations may range up to thirty-fold [23]. After 6 hours 6-TG becomes undetectable in plasma [24, 25], whereas it is rapidly transported into the cell in which 6-TG is immediately metabolized to 6-TGN.

We found that 6-TGN concentrations showed large interpatient variability. 6-TGN concentrations reached steady state after 4 weeks, suggesting a half-life of approximately 5 days. Twenty four-hour-curves showed almost constant 6-TGN concentrations. At steady state there was a five-fold range in 6-TGN concentrations, which is considerable however less pronounced than the range in 6-TGN originating from AZA or 6-MP [17]. Theoretically, this was to be expected as 6-TGN derived from 6-TG are formed in one single step compared to multiple steps when originating from AZA and 6-MP. Also, 6-TG is less affected by TPMT and a poor substrate for xanthine oxidase (XO) when compared to 6-MP [25] and genetic polymorphisms of these metabolizing enzymes are of less influence. Despite this fact, Herrlinger and colleagues identified one patient with TPMT heterozygosity (TPMT*1S/*3A) with bone marrow toxicity on 6-TG after a dose escalation from 40 to 80 mg/day in a cohort of 26 CD patients [20]. Furthermore,

6-TG caused severe and prolonged pancytopenia in an 8 year-old boy suffering from acute lymphoblastic leukemia with inherited TPMT deficiency [26]. In our cohort all genotyped subjects possessed wild-type alleles, so the high variability in metabolite levels could not be explained by genetic polymorphisms. Unfortunately, in half number of patients with 6-TG-intolerance TPMT genotype was not assessed and conclusions about the contribution of pharmacogenetics can not be drawn.

In this study, drug dose in milligrams per kilogram bodyweight did not correlate with 6-TGN concentrations. In a previous study with 6-MP we were not able to demonstrate correlation between drug dose and 6-TGN concentrations either [17].

6-TGN concentrations were considerably higher when compared with those in patients receiving standard treatment with AZA (2-3 mg/kg/d) or 6-MP (1-2 mg/kg/d). Similar results were found by others: four to nine-fold higher concentrations are reported on daily doses from 10-80 mg [11, 12, 20]. Despite 6-TGN concentrations well above the proposed therapeutic upper limit of approximately 500 picomoles/ 8×10^8 RBCs in patients using AZA or 6-MP, no myelotoxicity occurred in our cohort of patients, here-with confirming the results of a still increasing number of studies [11-14]. It was speculated that 6-TG derived 6-TGN are biochemically different from 6-MP derived 6-TGN [11]. A more convincing explanation was offered by Lancaster and colleagues: despite the accumulation of significantly higher erythrocyte 6-TGN concentrations for 6-TG compared with 6-MP, the accumulation of leukocyte 6-TGN in patients taking 6-TG was similar to the range of leukocyte 6-TGN in patients taking 6-MP [27]. In other words, when correlating intracellular 6-TGN to efficacy and myelotoxicity, RBC 6-TGN concentrations will be higher for patients taking 6-TG than in those taking 6-MP, whereas this is not the case in the putative target-tissue, the leukocytes.

In addition to this explanation, 6-methylmercaptapurine ribonucleotides (6-MMPR), which arise from 6-MP but not from 6-TG, are believed to contribute to the immunosuppressive and myelotoxic effects by inhibition of de novo purine synthesis [28]. The methylated 6-TGN, the 6-methylthioguaninenucleotides (6-MTGN), also inhibit the first step in de novo purine synthesis and consequently also have cytotoxic potential [20]. The 6-MTGN however, were not assessed in present study.

No hepatotoxicity occurred in the observed period of time. In a former study with patients on 6-MP treatment, hepatotoxicity correlated with 6-methylmercaptapurine (6-MMP) concentrations above 5700 picomoles/ 8×10^8 RBCs [16]. 6-MMP concentrations in our cohort on 6-TG treatment were undetectable as we demonstrated before in a short-term safety assessment [12]. This is an obvious finding because 6-MMPR are not formed when 6-TG is given instead of 6-MP (see figure 1). Recently, Dubinsky and colleagues discouraged using 6-TG in IBD because of serious liver injury [15, 29]. Disturbing high frequencies of NRH were reported in an IBD population treated with 6-TG. Steady state 6-TGN concentrations measured in Dubinsky's population (~ 1250 picomoles/ 8×10^8 RBCs) were significantly higher than in our population (~ 850 picomoles/ 8×10^8 RBCs). 6-TG induced NRH may in fact be dependent of the reached 6-TGN concentrations.

In one patient amylase and lipase levels temporary increased, but no pancreatitis developed and levels normalized without discontinuation of 6-TG. It is at least questionable whether this temporary elevation of enzymes was caused by 6-TG, because in AZA- or 6-MP-treated patients it is thought to be an idiosyncratic reaction, independent of drug dose or metabolite levels [30]. That is, dose-independent drug related side effects cannot disappear without drug withdrawal.

Four patients discontinued 6-TG within the observed 8 weeks, mainly due to the same adverse events as previously shown on AZA or 6-MP. 6-TGN concentrations were measured in 3 of 4 patients. In the patients with a probable and obvious relationship between 6-TG use and the occurrence of adverse events, 6-TGN levels were below the mean steady state concentration, herewith making a relationship between 6-TGN concentrations and discontinuation of 6-TG very unlikely.

Although our study was not primarily designed to study drug efficacy, we compared 6-TGN concentrations in patients with active disease with those in patients in remission after 8 weeks of 6-TG treatment. Mean 6-TGN concentrations in both groups did not significantly differ. Similar results were reported by Herrlinger and colleagues [20]. Besides, our data do not support the proposed therapeutic lower limit of 1300 picomoles/ 8×10^8 RBCs in IBD patients on 6-TG treatment [31]. All the patients who achieved remission in the observed period had 6-TGN concentrations below the proposed value. These findings are supported by the results from another study in which lower therapeutic target 6-TGN levels were proposed [14].

In conclusion, in IBD patients on 6-TG treatment, large interindividual differences in metabolite concentrations occur. In our population, we could not demonstrate a clear relationship between 6-TGN concentrations on one hand and toxicity and efficacy on the other like in AZA- and 6-MP-treated patients. It may be advisable however not to exceed 6-TGN concentrations of 1000 picomoles/ 8×10^8 RBCs hereby reducing the risk of developing NRH and veno-occlusive disease subsequently.

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Chapter 7

6-Thioguanine seems promising in azathioprine- or 6-mercaptopurine-intolerant inflammatory bowel disease patients

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Abstract

6-Mercaptopurine (6-MP) and azathioprine (AZA) have proven efficacy in the treatment of inflammatory bowel disease (IBD). However, adverse events leading to discontinuation may occur in 10-20% of patients. The efficacy of AZA and 6-MP is based on formation of their active metabolites, the 6-thioguaninenucleotides (6-TGN). Therefore, 6-thioguanine (6-TG), an agent more directly leading to formation of 6-TGN and until recently only used in patients suffering from leukemia, may be an alternative in case of AZA- or 6-MP-intolerance. The purpose of our study was to assess short-term safety of 6-TG.

Thirty two IBD patients with previously established AZA- or 6-MP-intolerance were treated with 6-TG as such in a dose of 20 mg (n=19) or 40 mg (n=13) once daily. Safety parameters were obtained at 0, 1, 2, 4 and 8 weeks after start of medication. Primary outcome measures were the ability to tolerate 6-TG and the occurrence of adverse events. Secondary outcome definitions included laboratory parameters.

Twenty six (81%) patients were able to tolerate 6-TG during the first 8 weeks. In 3 of 6 patients, side effects leading to discontinuation were probably (n=2) or obviously (n=1) 6-TG related. No clinically relevant hematological events or hepatotoxicity occurred in the observed period. Steady state 6-TG levels were significantly higher on 40 mg (1621 ± 828 picomoles/ 8×10^8 RBC) than on 20 mg once daily (937 ± 325 picomoles/ 8×10^8 RBC, $p < 0.001$).

6-TG treatment seems promising in AZA or 6-MP intolerant IBD patients. However, long-term safety and efficacy have yet to be determined.

Introduction

Thiopurine analogues 6-mercaptopurine (6-MP) and its prodrug azathioprine (AZA) are widely used in the second-line treatment of inflammatory bowel disease (IBD). These immunosuppressive agents have proven to be effective for both inducing and maintaining long-term remission of Crohn's disease (CD) and ulcerative colitis (UC) [1-3]. AZA is rapidly converted to 6-MP by a non-enzymatic reaction, which is further metabolized to the pharmacological active 6-thioguaninenucleotides (6-TGN) and 6-methylmercaptopurine (6-MMP). 6-TGN are purine nucleotides, which are incorporated into DNA, inducing cytotoxicity and immunosuppression [4]. In patients treated with 6-MP it is shown that red blood cell (RBC) 6-thioguanine (6-TG) levels correlated with drug efficacy and bone marrow suppression and 6-MMP levels, on the other hand, correlated with hepatotoxicity [5]. In patients treated with AZA or 6-MP the lower limit therapeutic 6-TG level has recently been established on 235 picomoles per 8×10^8 RBC, whereas 6-MMP levels above 5700 picomoles per 8×10^8 RBC were associated with hepatotoxicity [6].

A drawback of AZA and 6-MP is the occurrence of side effects. Adverse events leading to discontinuation may occur in approximately 10-20% of patients [7-9]. In general, adverse events may be distinguished in hypersensitivity reactions and dose dependent events. Hypersensitivity reactions to thiopurines or their metabolites occur mostly within 3-4 weeks and result in immune-mediated symptoms such as fever, rash, and arthralgia [10, 11].

Furthermore dose-dependent, pharmacological explainable reactions characterized by myelosuppression, increased susceptibility to infections and hepatotoxicity may occur. These reactions seem to be directly associated with high RBC 6-TG or 6-MMP levels and often reveal themselves in a subsequent stage.

Toxicity due to the 6-TG metabolite may be explained by polymorphisms in the gene encoding thiopurine S-methyltransferase (TPMT), the enzyme that methylates 6-MP to 6-MMP. In case of reduced enzyme activity toxic 6-TG levels may be reached on regular doses of AZA or 6-MP [12]. Alternatively, some patients develop high 6-MMP levels, whereas 6-TG levels remain subtherapeutic. In these patients the available 6-MP is largely transformed into 6-MMP due to preferential metabolism through TPMT, while the enzymatic pathway through hypoxanthine phosphoribosyl transferase (HPRT) leading to the formation of 6-TGN is hardly exploited. Recently, Dubinsky and colleagues successfully treated 7 out of 10 non-responding IBD patients with preferential 6-MMP formation on 100 mg 6-MP with 6-TG 40 mg once daily. In these patients 6-MMP levels were undetectable and no hepatic or hematological toxicity was observed [13]. Until recently 6-TG as such only was used in patients suffering from lymphoblastic leukemia.

Theoretically, therapy with 6-TG is a good alternative in patients with AZA- and 6-MP-intolerance of any kind. The idea of using a metabolite at the end of the enzymatic pathway seems to be an attractive approach. In this study we prospectively studied 6-TG short-term safety in AZA and 6-MP intolerant IBD patients.

Methods

Patient Selection

Patients aged between 18 and 75 years attending the out-patient clinics of Maasland Hospital Sittard, University Medical Center Nijmegen or Rijnstate Hospital Arnhem were eligible for the study if CD or UC was diagnosed for more than 6 months and an established AZA- or 6-MP-intolerance was observed. Exclusion criteria were: pregnancy or expected pregnancy within 6 months, inadequate contraception in women, lactation, presence of active infection, history of tuberculosis, HIV, hepatitis B or C, severe pancreatitis (necrotizing pancreatitis or pancreatitis leading to multi organ failure), malignancy, ongoing treatment with other immunosuppressive drugs like cyclosporine, methotrexate, thalidomide or infliximab, impaired renal function (serum creatinin > 2 times normal upper limit), impaired hepatic function (> 2 times normal upper limit) and persistent bone marrow suppression.

Study Design

The trial design was a prospective open-label multicenter study. At entry patient demographics and anamnesis were recorded and medication use during the past year was obtained from the community pharmacy. 6-TG (Lanvis™, tablet 40 mg, GlaxoWellcome) was administered orally once daily in the evening in a dose of 20 mg (2 centers) or 40 mg (1 center). Concomitant medication was continued. Safety parameters were obtained at 0 (base-line), 1, 2, 4 and 8 weeks after start of medication. Blood was drawn each visit for measurement of RBC 6-TG and 6-MMP levels, alanine transaminase (ALT), aspartate transaminase (AST), bilirubins, amylase, lipase, leukocytes (plus differentiation), platelets and hemoglobin (Hb). Dose-adjustments of 6-TG were carried out by the attending physician based on one or more of the parameters mentioned above.

Ethics

The protocol was approved by the local medical ethics committee and in accordance with the Helsinki Declaration. Informed written consent was obtained before enrolment.

Outcome Measures

Primary outcome measures were the ability to tolerate 6-TG and the occurrence of adverse events during 8 weeks of 6-TG treatment. The relationship of the adverse event with the use of 6-TG was established by the following method: unrelated, no temporal relation and other etiology likely; possibly related, potential temporal relation and other etiologies possible; probably related, potential temporal relation and other etiologies unlikely; related, clear temporal relation not otherwise explained. Secondary outcome definitions included RBC 6-TG and 6-MMP levels, the occurrence of hematological events (leukocyte count < $4.0 \times 10^9/l$, platelet count < $100 \times 10^9/l$) and the occur-

rence of hepatic toxicity (ALT > 80 U/l, AST > 80 U/l, bilirubins > 40 μ mol/l, amylase > 220 U/l, or lipase > 120 U/l (elevations > 2 times normal upper limit)).

Analytical Procedures

Blood samples were taken at least 12 hours after medication intake. The samples were centrifuged to isolate erythrocytes and after washing with PBS buffer solution, erythrocyte counts were done. Samples were stored at -20°C until required. RBC 6-TG and 6-MMP levels were measured in the laboratory of the Department of Clinical Pharmacy, Maasland Hospital Sittard, using a slightly modified assay previously reported by Lennard and colleagues [14]. In brief, the thiopurines are separated by reversed-phase HPLC and quantified using UV-detection. For measurement of intracellular thionucleotides the free base is obtained by acid hydrolysis of the nucleotide back to the purine. The resulting purines are extracted from the biological matrix by forming a phenylmercury adduct into dichloromethane. During back-extraction with hydrochloric acid the adduct is split and the free thiopurine liberated into the acid layer once again. The run-to-run coefficient of variation was 6.6% and 9.7% for 6-TG and 6-MMP respectively. The detection limit of the assay was 30 picomoles/ 8×10^8 RBC for 6-TG and 300 picomoles/ 8×10^8 RBC for 6-MMP.

Statistical analysis

Data are expressed as means $\pm$ standard deviation. Significance was evaluated by the Student *t* test for paired or independent data, *p* values < 0.05 were considered significant. Pearson's correlation was used to test the relationship between the measured parameters. SPSS for Windows (version 10.0.7) software was used to perform statistics.

Role of the funding source

This study was not sponsored by external sources.

Results

Patients

Thirty two patients were enrolled, of whom 7 (22%) were male and 25 (78%) were female. The mean age at entry was 37 years (range 19-64). Twenty two patients (69%) were suffering from CD and in 10 patients (31%) UC was diagnosed. Twenty one patients (66%) emerged AZA-intolerant, 5 (16%) could not tolerate 6-MP and 6 (19%) patients showed intolerance for both thiopurines. AZA- or 6-MP-intolerance was established with rechallenge in 12 patients (38%). The adverse events leading to discontinuation of AZA or 6-MP included total malaise (41%), nausea & vomiting (38%), fever (25%), arthralgia (13%), amylase elevations (13%), epigastric pains (13%), pancreatitis (amylase elevations with symptoms) (9%), indefinite symptoms (9%), headache (6%), diarrhea (3%), myalgia (3%), erythema nodosum (3%), edema (3%) and

pruritus (3%). In 10 patients AZA- or 6-MP-intolerance manifested itself within 2 weeks, in 5 within 1 month, in 6 within 2 months and in 6 after 2 months. In 4 patients time to intolerance was not documented. The initial dose of 6-TG was 20 mg daily in 19 patients and 13 received 40 mg a day. The mean initial 6-TG dose was 0.40 ± 0.03 mg/kg/day. The mean 6-TG dose during the 8-week-period was 25.9 mg/day (range 10-40).

Primary Outcomes

Twenty six (81%) of the 32 subjects were able to tolerate 6-TG 20 or 40 mg 1dd during the first 8 weeks. In 6 patients (19%) 6-TG was discontinued because of adverse effects. Data of the 6-TG-intolerant patients are summarized in table 1.

In 2 patients indefinite joints pains occurred on 40 mg 1dd that resolved after dose-reduction to 20 mg 1dd. One patient temporary stopped after developing nausea on 40 mg 1dd that disappeared after restart on 20 mg 1dd. Another patient had a dry cough and was not feeling well on 20 mg 1dd which resolved after dose-reduction to 20 mg every 2 days.

Secondary Outcomes

RBC 6-TG and 6-MMP levels

Base-line RBC 6-TG levels were negative in all but 4 patients who just recently stopped AZA (n=2) or 6-MP (n=2) therapy. Steady state 6-TG levels were significantly higher on 40 mg 1dd (1621 ± 828 picomoles/ 8×10^8 RBC) than on 20 mg 1dd (937 ± 325 picomoles/ 8×10^8 RBC, $p:0.001$) according on treatment analysis. RBC 6-TG levels were considered to reach steady state after 4 weeks, having a half life of approximately 5 days [15]. RBC 6-MMP levels were undetectable at all times during treatment with 6-TG, except at base-line in the 4 patients mentioned before. Correlation was found between dose and RBC 6-TG level (0.70, $p:0.01$). The mean RBC 6-TG level increased most during in the first part of the 8-week-period and levelled out between 4 and 8 weeks (figure 1).

Hematological events

No clinically relevant myelotoxicity occurred in the 8-week-period. However, the mean leukocyte count decreased significantly from base-line ($11.4 \pm 3.9 \times 10^9/l$) to 8 weeks of 6-TG treatment ($10.0 \pm 4.1 \times 10^9/l$, $p:0.008$). Leukocyte counts were never below $4.0 \times 10^9/l$, no leukopenia was observed. RBC 6-TG levels showed moderate correlation with leukocyte counts (-0.29 , $p:0.01$). A minor thrombocytopenia was observed in one patient (platelet count: $130 \times 10^9/l$). In this cohort of 32 patients, platelet count neither decreased nor correlated with RBC 6-TG level in the observed period. In addition no change in Hb was established.

Table 1. Adverse events leading to discontinuation of 6-thioguanine treatment

No	Initial dose (in mg)	Final dose (in mg)	Time to AE (in days)	Character of AE	Relationship AE/6-TG
1	20	20	24	nausea*, diarrhea*, stomach cramps*	probably related
2	20	10	42	total malaise*	possibly related
3	20	20	7	fever*, erythema nodosum*, arthralgia*	related
4	20	10	7	nausea*, stomach cramps, headache*	probably related
5	20	20	35	rigid fingers	possibly related
6	20	40	10	indefinite stomach symptoms	possibly related

AE, adverse events; 6-TG, 6-thioguanine; *) same AE as previously shown on AZA or 6-MP.

Figure 1. Mean red blood cell (RBC) 6-thioguanine (6-TG) levels during the first 8 weeks of 6-TG treatment.

Hepatotoxicity

The ALT level was elevated > 80 U/l in one patient, but this was the case at base-line already. The mean ALT level did increase significantly from 19.8 ± 19.9 U/l at base-line to 23.5 ± 25.7 U/l at 8 weeks, even though not clinically relevant. Amylase level was above 220 U/l (234 U/l) in one patient. Moderate correlation was found between RBC 6-TG level and amylase level (0.30, $p: 0.01$). No AST, bilirubins or lipase elevations were observed and mean levels did not change during the 8 weeks. No correlation was found between RBC 6-TG level on the one hand and ALT, AST, bilirubins or lipase for another.

Discussion

6-TG was well tolerated during the first 8 weeks of treatment in 81% of patients with previously established AZA- or 6-MP-intolerance. In some patients intolerance for AZA or 6-MP manifested itself after 8 weeks. Therefore, we must be reserved in drawing firm conclusions as the number of patients with adverse events on 6-TG may increase over time. Six patients (19%) were unable to complete the 8-week-period on 6-TG. The relationship of adverse events with 6-TG was unclear but possible in 3 of the 6 patients whose 6-TG treatment ended prematurely. In 2 patients the side effects were probably 6-TG related. In these patients, adverse events on 6-TG were very similar to the previous side effects on AZA or 6-MP, suggesting a causal role for 6-TGN in the development of these events. Moreover, these adverse events resolved after discontinuation of 6-TG. In one patient the side effects were obviously 6-TG related, being identical and occurring at exactly the same time as previously shown on AZA. Also, in this patient adverse

events disappeared with discontinuation of 6-TG. In 4 other patients transient side-effects occurred, which all dissolved after a 50% dose-reduction (3 patients from 40 mg to 20 mg daily and 1 patient from 20 mg to 10 mg daily).

Mean RBC 6-TG levels were high in comparison with 6-TG levels measured in AZA- or 6-MP-treated patients. In the latter group we previously measured mean 6-TG levels of 246 and 264 picomoles/ 8×10^8 RBC on AZA 100 mg 1dd and 6-MP 100 mg 1dd respectively [16]. The mean steady state 6-TG levels after 6-TG as such once daily (20 mg: 937 picomoles / 8×10^8 RBC; 40 mg: 1621 picomoles/ 8×10^8 RBC) were approximately four- to sevenfold higher. This is consistent with the observations of Dubinsky and colleagues [13]. In present study, RBC 6-TG levels were well above the proposed lower limit of 235 picomoles/ 8×10^8 RBC for therapy with AZA and 6-MP, which was associated with efficacy. However, it remains unclear whether the same level should be used when 6-TG is given as such. Therefore, efficacy data from prospective randomized trials are needed.

RBC 6-TG level correlated significantly with the dose given. However, major interindividual differences occur as is seen with the other thiopurines [17, 18]. Therapeutic drug monitoring (TDM) may be a helpful tool in dosing 6-TG. In this study we used TDM to control patient compliance and to prevent extremely high RBC 6-TG levels (> 2500 picomoles/ 8×10^8 RBC). The presumed period necessary to reach steady state of 4 weeks seems reliable, as the RBC 6-TG concentrations levelled out somewhere between 4 and 8 weeks after start of medication. In patients treated with AZA or 6-MP, myelotoxicity is a major concern, which seems to be associated with the RBC 6-TG level. However, in the present study, despite high RBC 6-TG levels no leukopenia was observed in our 6-TG-treated patients. Although the mean leukocyte count decreased from base-line ($11.4 \times 10^9/l$) to 8 weeks of 6-TG treatment ($10.0 \times 10^9/l$), the clinical relevance is restricted as there still is a large margin to the critical lower limit of $4.0 \times 10^9/l$. A moderate inverse correlation of leukocyte count with RBC 6-TG level was observed and because 6-TG levels seemed to have reached a steady state, further leukocyte count reduction is unlikely. The fact that no leukopenia occurred may be explained by the absence of the 6-MMP metabolite, which seems to have antiprolerative properties of its own [5, 19]. No 6-MMP is formed, when a patient is given 6-TG as such. In AZA- or 6-MP-treated patients however, substantial 6-MMP levels arise, which may contribute to myelotoxicity. The role of 6-MMP needs further elucidation. In spite of absence of leukopenia in this cohort of patients, alertness remains mandatory as leukopenia is a serious complication.

Mild thrombocytopenia occurred in one patient. This patient had a history of open heart surgery and was still receiving antithrombotic prophylaxis during 6-TG treatment. Thrombocyte count normalized without 6-TG dose-reduction and therefore a relationship between thrombocytopenia and 6-TG treatment seems unlikely.

No serious hepatotoxic events occurred in the observed period, not even in the 5 patients which showed earlier hepatic or pancreatic events (amylase- or ALT elevations) on AZA or 6-MP. In one patient the ALT level was elevated, but as this already was the

case before inclusion this elevation can not be accounted for by 6-TG treatment. Furthermore, in this patient ALT level decreased with ongoing therapy with 6-TG. Although mean ALT levels builded up during 6-TG treatment to a value of 23.5 U/l, the increase was of no clinical importance, as this is far from the standard upper limit of 40 U/l. In another patient the amylase level was slightly elevated which normalized after 6-TG dose was reduced from 40 mg to 20 mg once daily. Three patients with non-severe pancreatitis within 4 weeks of AZA or 6-MP treatment, did not develop pancreatitis in 8 weeks of 6-TG treatment. Unfortunately in only one of these patients a RBC 6-MMP level was previously measured on 6-MP treatment and this level was substantial (4893 picomoles/8x10⁸ RBC after 2 weeks on 6-MP 50 mg once daily). This may indicate a role for 6-MMP in the development of pancreatitis in this patient. Although high RBC 6-MMP levels are often detected in patients developing pancreatitis, sometimes pancreatitis ensues despite the absence of 6-MMP [5, 6]. The role of 6-MMP levels in the occurrence of pancreatitis is still unclear.

Treatment with 6-TG seems a promising alternative in AZA- and 6-MP-intolerant IBD patients. The results on short-term safety are encouraging for the future. However, long-term safety in IBD has yet to be determined as adverse events like veno-occlusive disease and portal hypertension are reported in 6-TG treatment of leukemia. Also efficacy has to be proven before 6-TG can be added to standard therapy. For future trials a dose of 20 mg once daily results in presumably adequate RBC 6-TG levels and seems well tolerated. The impact of high 6-TG levels during a longer period of time remains unclear and merits further exploration for both efficacy- and safety reasons.

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Chapter 8

On tolerability and safety of a maintenance treatment with 6-thioguanine in azathioprine- or 6-mercaptopurine-intolerant inflammatory bowel disease patients

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Abstract

The use of 6-thioguanine may lead to an avoidance of toxic pathways as it is directly converted to the pharmacologically active metabolites (the 6-thioguaninenucleotides (6-TGN)).

The aim of our study was to determine the tolerability and safety profile of a low-dose maintenance therapy with 6-TG in azathioprine (AZA) or 6-mercaptopurine (6-MP) intolerant IBD patients over a treatment period of at least one year. The data were obtained by database analysis.

Twenty out of 95 (21%) patients discontinued 6-TG (mean dose 24.6 mg) within one year. Reasons for discontinuation were GI-complaints (31%), malaise (15%) and hepatotoxicity (15%). Hematological events occurred in three patients, one discontinued treatment. In the 6-TG tolerant group 9% (7/75) could be classified as hepatotoxicity. An abdominal ultrasound was performed in 54% of patients, one patient had splenomegaly.

The majority of AZA- or 6-MP-intolerant IBD patients (79%) is able to tolerate maintenance treatment with 6-TG (dosaged between 0.3 to 0.4 mg/kg/day). 6-TG may still be considered as an escape maintenance immunosuppressant in this difficult to treat group of patients, taking into account potential toxicity and efficacy of other alternatives. The recently reported hepatotoxicity is worrisome and 6-TG should therefore only be administered in prospective trials.

Introduction

Until now, no definite medical or surgical therapy is available for the treatment of inflammatory bowel disease (IBD) as the etiology remains essentially unknown. Therefore, the aims of medical therapy in IBD are to relieve symptoms and to prevent long-term complications. Maintenance therapy should preferably be efficient, safe and cost-effective. The immune modulating thiopurines 6-mercaptopurine (6-MP) and its pro-drug azathioprine (AZA) have proven efficacy in active IBD and in maintenance of an induced clinical remission [1]. These thiopurines are prescribed on a large scale in IBD. However, issues concerning delayed onset of activity, refractoriness and toxicity have limited the general use of AZA and 6-MP. In previous reports, up to 20% of patients discontinued AZA or 6-MP prematurely due to adverse events [2]. The metabolism of thiopurines has been partly elucidated in recent years (figure 1).

The toxicity profile depends at least partly on the generated metabolites of AZA and 6-MP [3]. At first, AZA is converted to 6-MP by a non-enzymatic reaction, which is then

Figure 1. Thiopurine metabolism. Azathioprine (AZA) is degraded to 6-mercaptopurine (6-MP) and nitromethylimidazole, via a non-enzymatic step. Three competing enzymes metabolize 6-MP. Xanthine oxidase (XO) inactivates 6-MP to thiouric acid (6-TUA). Thiopurine S-methyltransferase (TPMT) methylates 6-MP, the formed 6-methylmercaptopurine (6-MMP) is associated with hepatotoxicity. By hypoxanthine phosphoribosyl transferase (HPRT) 6-MP is catalysed to 6-thioinosine monophosphate (6-TIMP), then via inosine monophosphate dehydrogenase (IMPD) to 6-thioxanthosine monophosphate (6-TXMP), ultimately leading to the pharmacologically active 6-thioguaninenucleotides (6-TGN) via the enzyme guanosine monophosphate synthetase (GMPS). The 6-TGN are methylated to 6-methylthioguaninenucleotides (6-MTGN). 6-TIMP can also be methylated to 6-methylthioinosine monophosphate (6-MTIMP). In a normal useless cycle 6-TIMP is phosphorylated by monophosphate kinase (MPK) to 6-thioinosine diphosphate (6-TIDP), subsequently by diphosphate kinase (DPK) to 6-thioinosine triphosphate (6-TITP) and ultimately back to 6-TIMP due to inosine triphosphate pyrophosphatase (ITPase). When ITPase activity is impaired or lacking, 6-TITP accumulates. 6-Thioguanine (6-TG) is directly converted by HPRT to the 6TGN. XO and TPMT metabolize 6-TG to 6-TUA and 6-methylthioguanine (6-MTG), respectively. ITPase has no known role in the metabolism of 6-TG.

converted by a multi-step enzymatic pathway into a number of active, inactive or toxic metabolites. The efficacy of AZA and 6-MP appears to be correlated with the formation of the 6-thioguaninenucleotides (6-TGN). During this complex metabolization process possible hepatotoxic metabolites are generated under influence of the enzyme thio-purine S-methyltransferase (TPMT). The methylation products of 6-MP, 6-methylmercaptapurine (6-MMP) and their ribonucleotides (6-MMPR), may be associated with hepatotoxicity [3]. Furthermore, flu-like symptoms, rash, pancreatitis and neutropenia induced by AZA or 6-MP have recently been related to mutations in the inosine triphosphate pyrophosphatase (ITPase) gene leading to accumulation of the proposed metabolite thio inosine triphosphate [4]. A possible strategy to avoid AZA or 6-MP induced toxicity is the administration of a thiopurine, which is metabolically closer to the 6-TGN. The thiopurine 6-thioguanine (6-TG) is an attractive candidate, which has the advantage of being directly converted to 6-TGN [5]. Therefore, treatment and safety outcomes might be less sensitive for intra- and interindividual metabolic variations. Furthermore, due to the relative simple metabolization, the number of possibly toxic metabolites is strongly reduced. Several open label studies in patients with IBD have shown promising efficacy and acceptable short-term toxicity of 6-TG [6, 7]. More recently, the use of 6-TG has been associated with the induction of nodular regenerative hyperplasia of the liver (NRH). The aim of the present study was to determine the tolerability and safety profile of a low-dose maintenance therapy with 6-TG in AZA or 6-MP intolerant IBD patients over a treatment period of at least one year.

Material and Methods

Patient selection

The study is a retrospective database analysis exploring the tolerability and safety of 6-TG over at least a one-year treatment period in AZA- or 6-MP-intolerant IBD patients, treated in participating university and district hospitals in the Netherlands. In each patient, the attending physician judged the indication for administration of 6-TG. Some patients have been described earlier in a short safety assessment of 6-TG [6]. Patients were eligible for the study if they met the following in- and exclusion criteria. Inclusion criteria were: age between 18 and 75 years, presence of confirmed CD or UC with an indication for immunosuppressive therapy but in whom standard AZA or 6-MP therapy failed due to adverse events. Immunosuppression was indicated in case of chronic active disease, corticosteroid dependency or recurrent disease. Exclusion criteria were pregnancy, lactation, presence of active infection, history of tuberculosis, HIV, hepatitis B or C, history of severe pancreatitis (necrotizing pancreatitis or pancreatitis leading to multi-organ-failure), ongoing treatment with concomitant immunosuppressive drugs (e.g. cyclosporine, methotrexate, thalidomide or infliximab), impaired renal function (serum creatinin > 2 times normal upper limit), impaired hepatic function (> 2 times normal upper limit) and persistent bone marrow suppression.

Study design

In all patients, 6-TG (Lanvis™, tablet 40 mg, GlaxoWellcome) was administered orally in a dose of 20 to 40 mg once daily based on the decision of the attending physician. The following data were collected: patient demographics, history of thiopurine exposure, type of thiopurine-intolerance, the use of concomitant medication, blood cell counts and liver enzymes. Dose of 6-TG, duration of 6-TG, occurrence of adverse events, blood cell counts and liver enzymes were reviewed after a minimum of 12 months after initiation of 6-TG treatment. During the described period the genotyping of TPMT became available and in a subgroup of patients the TPMT status was determined. Red blood cell (RBC) 6-thioguanine nucleotides (6-TGN) levels were determined at least four weeks after giving a stable dose in order to obtain steady-state levels of 6-TGN. Dose adjustments were left to the discretion of the attending physician, but when 6-TGN levels were above 1000-1500 picomoles/ 8×10^8 RBC, dose reduction was contemplated. In case of a leukocyte count below 3.5×10^9 /l, dose reduction was advocated and in case of severe leucopenia (below 1.0×10^9 /l) 6-TG had to be discontinued. Additionally, in order to explore signs of hepatotoxicity, an abdominal ultrasonography was advised after at least one-year 6-TG administration. Special attention was paid to splenomegaly, signs of portal hypertension and hepatic changes.

Outcome Measurements

Primary outcome measures were the ability to tolerate 6-TG and the occurrence of adverse events leading to discontinuation of 6-TG over the one-year period. The relationship of any adverse event with the use of 6-TG was established by the following method: unrelated, no temporal relation and other etiology likely; possibly related, potential temporal relation and other etiologies possible; probable related, potential temporal relation and other etiologies unlikely; related, clear temporal relation not otherwise explained. Secondary outcome measures were the occurrence of hematological events (defined as leukocyte count $< 4.0 \times 10^9$ /l or platelet count $< 100 \times 10^9$ /l), the occurrence of hepatotoxicity (defined as a rise of at least two times the upper normal limit of a single liver enzyme level), pancreaticotoxicity (serum amylase > 220 U/l) and signs of liver related abnormalities on the abdominal ultrasound. The following data concerning disease activity were analysed as well: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), serum albumin and a global physician assessment score (features were same, better or worse).

6-TG metabolite monitoring and genotyping of TPMT

Blood samples for 6-TGN measurements were obtained at least 4 weeks after the onset of a stable 6-TG dose. The samples were centrifuged to isolate erythrocytes and after washing with PBS buffer solution, erythrocyte counts were done. Samples were stored at -20°C until required. RBC 6-TGN levels were measured in the laboratory of the Department of Clinical Pharmacy, Maasland Hospital Sittard, using a proprietary modified previously published assay [8]. The lower limit of quantification of the assay was 30

picomoles/ 8×10^8 RBC for 6-TGN levels with a run-to-run coefficient of variation of 6.6%. Extra blood samples were drawn once during the one-year period to assess TPMT (G238C, G460A and A719G, i.e. TPMT*1, TPMT*2, TPMT*3A, TPMT*3B, TPMT*3C) genotypes in a sub-group of patients, independent of the biochemical or clinical status. The genotyping was performed at the Department of Gastroenterology and Hepatology at the Academic Medical Center in Amsterdam.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation. Significance was evaluated by the Student t test for paired or independent data; P values of less than 0.05 were considered significant. Pearson's correlation was used to determine relationships between parameters. A χ^2 test was used to determine the significance between the TPMT genotype and 6-TG-intolerance. SPSS for windows version 11.0 was used for statistical analysis.

Results

Patients

In ninety five patients treatment with 6-TG was initiated in the period June 2001 to July 2003. Fifty eight patients (61%) were female (mean age: 40 years, range: 20-74) and 37 (39%) were male (mean age: 47 years, range: 20-71). Forty-two (44%) patients were diagnosed with UC compared to 53 (56%) patients with CD. All patients were intolerant to AZA, 6-MP or both. The adverse events leading to discontinuation of AZA or 6-MP use were gastrointestinal complaints, general malaise, allergic reactions, hepatotoxicity or myelotoxicity. The patients characteristics are given in detail in table 1. The mean initial 6-TG dose was 24.6 mg (range 20-40 mg). The mean initial 6-TG dose adjusted to bodyweight was 0.37 mg/kg (sd 0.16).

Primary outcomes

Seventy five (79%) of the 95 patients were able to tolerate 6-TG during one year use. In 20 (21%) patients the administration was discontinued due to side effects. The 20 intolerant patients (nine females and eleven males) encountered 26 side effects. The data concerning the adverse events leading to withdrawal and the relationship with 6-TG use are summarized in table 2. No mortality was reported. The mean 6-TG dosage was 0.30 mg/kg in the tolerant group compared to 0.34 mg/kg in the intolerant group (NS).

Secondary outcomes

Hematological events

Three patients had signs of myelosuppression. One patient had a leukocyte count of $3.2 \times 10^9/l$ and a platelet count of $70 \times 10^9/l$ after 50 days of 6-TG use (20 mg). Subsequently, 6-TG was discontinued. The second patient had a leukopenia (leukocytes

Table 1. Patient characteristics

	n=95
Sex (M/F)	37/58
Age (yrs) (mean + range)	43 (20-74)
Disease (CD/UC)	53/42
Duration of IBD at start 6-TG (yrs) (mean + sd)	10.4 (9.4)
AZA-intolerance	78 (82%)
6-MP-intolerance	4 (4%)
AZA- and 6-MP-intolerance	13 (14%)
AZA- or 6-MP-rechallenge	36 (38%)
Adverse events on AZA or 6-MP	
gastrointestinal complaints	30%
general malaise	20%
allergic reactions	14%
pancreaticotoxicity	10%
hepatotoxicity	6%
myelotoxicity	5%
other (e.g. myalgia or alopecia)	14%
6-TG dosage at start (mg) (mean + range)	24.6 (20-40)

M, male; F, female; CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; 6-TG, 6-thioguanine; AZA, azathioprine; 6-MP, 6-mercaptopurine.

$3.5 \times 10^9/l$) and a third patient had a thrombocytopenia (platelets $87 \times 10^9/l$) at one year measurements, both did not discontinue 6-TG. All other patients, both tolerant and intolerant to 6-TG, had platelet and leukocyte counts above the set lower limits. However, the mean platelet count decreased significantly from baseline ($309 \times 10^9/l$) to one year of 6-TG treatment ($290 \times 10^9/l$, $P=0.033$). The mean leukocyte count decreased as well (from 11.9 to $7.8 \times 10^9/l$) though not significantly. The 6-TGN level did not correlate with the decrease in both leukocytes and platelets counts. Hemoglobin levels remained unchanged in both patient groups.

Hepatotoxicity and pancreaticotoxicity

In the 6-TG-tolerant group the ASAT, ALAT, GGT and AF levels were determined in 65%, 71%, 60% and 68% of patients at one year 6-TG use, respectively. The mean levels of ASAT, ALAT, GGT and AF were 24.7 U/l (range 6-118), 24.5 U/l (range 5-206), 48 U/l (range 6-535) and 84 U/l (range 42-322), respectively. A significant increase in liver enzymes during treatment in the tolerant group was not established. No correlation was found between the 6-TGN level and ALAT, ASAT, GGT or AF levels. At one year use, in nine percent of patients (7/75) hepatotoxicity occurrence on 6-TG treatment could be classified. Four patients (4/20) of the intolerant group discontinued 6-TG use due to hepatotoxicity. All had normal liver tests at entry and three of these patients encountered allergic reactions but no hepatotoxicity on the prior AZA therapy. Three patients had a serum amylase level above 220 U/l before the start of 6-TG that all became normal during treatment. One patient developed a transitory symptomatic

Table 2. Adverse events (n=26) leading to discontinuation of 6-TG use in 20 patients

AE on 6-TG	Frequency	Mean time to AE (days)	Relationship AE/6-TG
gastrointestinal complaints	8/26 (31%)	43	probably related 6/8; possibly related 2/8
hepatotoxicity	4/26 (15%)	182	probably related 2/4; possibly related 1/4; unrelated 1/4
myelodepression	1/26 (4%)	50	related
pancreaticotoxicity	1/26 (4%)	103	possibly related
general malaise	4/26 (15%)	51	probably related 3/4; possibly related 1/4
allergic reactions	1/26 (4%)	1	related
other AE	7/26 (27%)	39	related 1/7; probably related 2/7; possibly related 4/7

AE, adverse event; 6-TG, 6-thioguanine

pancreatitis with a serum amylase level of 430 U/l during 6-TG treatment and discontinued treatment.

Abdominal ultrasonography

In 51 patients (54%) an abdominal ultrasonography was performed after at least one year 6-TG use. Five ultrasounds were considered as abnormal; three patients had steatosis, one patient had a choledocholithiasis and the last patient had a hydrops of the gallbladder and a splenomegaly (spleen size 13 cm). The latter two patients were classified as hepatotoxicity on 6-TG treatment.

Efficacy of 6-TG

No significant decrease in CRP (15 to 11 mg/l) or ESR level (15 to 14 mm) was established in the 6-TG tolerant group. The albumin level increased (37.6 to 40.1 g/l) significantly in the tolerant group ($p=0.002$). A significant decrease of albumin level (38.5 to 36.6 g/l) was established in the intolerant group ($p=0.03$). The global physician score was determined at one year 6-TG use in 85% (64/75) of the tolerant patients. Forty-seven patients (73%) were classified as better, 15 patients (23%) as the same and four patients (6%) as worse during 6-TG treatment.

6-TG metabolite monitoring and genotyping of TPMT

The mean steady state 6-TGN level, determined in 63% (47/75) of the tolerant patients, was 540 picomoles/ 8×10^8 RBC (sd 245, range 0 – 1404). The difference in 6-TGN levels between tolerant and intolerant patients was not significant. No statistical significant correlations were established between laboratory parameters (leukocytes, platelets, hemoglobin, AF, GGT, ASAT and ALAT) and 6-TGN level. The TPMT genotyping was performed in 51 patients (54%). Two patients (3.9%) had one mutant non-functional allele (TPMT*3A and TPMT*3C) and both patients were intolerant for 6-TG (myelodepression and hepatotoxicity) ($p=0.003$). The 6-TGN level was not de-

terminated in both patients. Patients with two mutant non-functional alleles of TPMT (homozygous mutants) were not found in our population.

Discussion

In this group of AZA- or 6-MP-intolerant IBD patients, we demonstrated that 6-TG in dosages of 0.3 to 0.4 mg/kg daily was well tolerated over a period of one year as a maintenance immunosuppressant in 79% of the patients. This result suggests that during the metabolism process of AZA or 6-MP metabolites are generated that induce side effects which at least in part are not generated during the 6-TG metabolism. No mortality was reported due to 6-TG use. When 6-TG was discontinued, the side effects leading to withdrawal resolved spontaneously. One patient developed a myelodepression that may be explained by the impaired TPMT activity, probably shunting 6-TG away from methylation by TPMT towards the formation of 6-TGN which have been associated with myelotoxicity. Unfortunately, no 6-TGN level was determined in this patient. The real incidence of 6-TG related histological liver abnormalities like NRH or veno-occlusive disease (VOD) in the present study remains unknown, as liver biopsies were not performed. However, the incidence of 6-TG related increase in liver enzymes probably will be lower than 7 out of 75 patients, because two patients already had abnormal liver tests before the start of 6-TG and one patient had a symptomatic choledocholithiasis.

In the present study we found a mean 6-TGN level of 540 picomoles/ 8×10^8 RBC in the tolerant group which is above the proposed therapeutic threshold of 250 picomoles/ 8×10^8 RBC and even higher than the proposed upper limit of efficacy (450 picomoles/ 8×10^8 RBC) under AZA or 6-MP therapy [3, 9]. This is consistent with previous observations [6, 7]. It remains to be elucidated whether the same 6-TGN limits should be used when 6-TG is administered. In our study 73% clinically benefited from 6-TG by using the global physician score and the mean albumin level increased significantly. However, due to the retrospective nature of the study, we must be reserved in drawing firm conclusions as no standard efficacy parameters as the Crohn's disease activity index (CDAI) or Truelove-Witts index were used. Despite the fact that we have not demonstrated a significant relationship between 6-TGN levels, laboratory results and adverse events, we believe that therapeutic drug monitoring may be a helpful tool in dosing 6-TG. Extremely high 6-TGN levels can be prevented and compliance can be monitored.

The TPMT status has been associated with the ability to tolerate AZA or 6-MP. Patients with impaired TPMT activity are more prone to develop a myelodepression [10]. However, our study demonstrates that in AZA- or 6-MP-intolerant patients only two patients (3.9%) had one mutant TPMT allele which is even lower than the incidence in the normal Caucasian population [11]. Interestingly, both patients did not develop a myelodepression on AZA therapy. This indicates that other metabolic pathways may

lead to toxicity of AZA or 6-MP as was recently demonstrated for the ITPase routing (Derijks et al., submitted for publication). However, both patients with one mutant TPMT allele discontinued the use of 6-TG. Unfortunately, in these patients no 6-TGN concentrations were measured before discontinuation of 6-TG. Possibly, an impaired TPMT activity leads to more 6-TG being metabolized by the enzymes hypoxanthine phosphoribosyl transferase or xanthine oxidase leading to higher 6-TGN and 6-thiouric acid levels, respectively (figure 1). The accumulation of these metabolites may lead to intolerance of 6-TG.

Recently, the use of 6-TG in IBD patients has been associated with the induction of NRH and VOD [12]. In the present study, we performed an abdominal ultrasonography after at least one year 6-TG use to screen for possible hepatotoxicity in 51 patients. Only one patient showed signs of portal hypertension indicated by an enlarged spleen. NRH has been associated with thrombocytopenia [12] and in our evaluation only two patients had platelets counts below $100 \times 10^9/l$. We are well aware of the fact that by using biochemistry and ultrasound outcomes, we probably underestimate the real incidence of 6-TG induced NRH, as the golden standard is based on histology. Additionally, liver tests abnormalities were shown not to be indicative for NRH. Conversely, it should be taken into account that AZA and 6-MP can induce NRH or VOD as well but performing liver biopsies during treatment with these compounds is not recommended in clinical practice.

Currently, the use of 6-TG in IBD is abandoned due to potential hepatotoxicity [12]. However, low dose 6-TG may still be considered as an escape maintenance strategy in AZA- or 6-MP-intolerant IBD patients whom are refractory for alternative therapies. The number of proven effective medical maintenance options is scarce for these patients. Methotrexate (MTX) has a reasonable toxicity profile and seems effective in CD, comparable with AZA or 6-MP [13] but the potential use of MTX in UC patients has yet to be proven. In addition, long-term use of MTX may be limited [14]. Cyclosporine seems to have no role as a maintenance immunosuppressive alternative in IBD [15]. Infliximab can be administered as a maintenance option in CD with acceptable toxicity and may be effective in treating UC. However, concomitant immunosuppressive therapy with thiopurines or MTX next to the infliximab therapy seems mandatory to reduce the immunogenic response [16].

Treatment with 6-TG is well tolerated in AZA and 6-MP intolerant IBD patients and seems to be effective. We believe that low dosed 6-TG (0.3 to 0.4 mg per kilogram daily) maintenance therapy may still be an escape option for this difficult to treat group of patients. The benefit of 6-TG use in this subgroup may balance its toxicity profile, especially taking into account the toxicity and efficacy of other therapeutic alternatives. Still, the reported hepatotoxicity is worrisome and 6-TG should therefore only be administered in prospective clinical trials.

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Part V

Conclusions and perspectives

Chapter 9

Thiopurines in inflammatory bowel disease: new strategies for optimization of pharmacotherapy?

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Introduction

One in every three inflammatory bowel disease (IBD) patients on thiopurines lacks efficacy and up 20% is forced to discontinue due to the occurrence of adverse events [1-6]. In the past 10-20 years, knowledge of both thiopurine pharmacology and pharmacogenetics has increased dramatically leading to the development of new strategies to improve efficacy and reduce toxicity of thiopurines in IBD. The overall goal of the research described in this thesis was to further increase basic knowledge of thiopurine pharmacology and determine whether or not the application of certain new strategies is an improvement in the management of IBD with thiopurines. These strategies included therapeutic drug monitoring (TDM), genotyping two crucial enzymes in thiopurine metabolism and the use of 6-thioguanine (6-TG) as such.

Therapeutic drug monitoring

TDM has proven its value in improving efficacy and reducing toxicity of many drugs like antibiotics (gentamicin), anti-epileptics (phenytoin), cardiovascular drugs (digoxin) and immunosuppressives (cyclosporine). In the nineties, several assays for the quantification of the active metabolites of the thiopurines, the 6-thioguaninenucleotides (6-TGN) and the 6-methylmercaptopurine ribonucleotides (6-MMPR), were developed [7-9].

Subsequently, numerous clinical studies were performed in which thiopurine metabolites were assayed [10-19]. At present, there is debate whether TDM is useful in the fine-tuning of thiopurines in IBD because conflicting data have been published [20, 21]. These controversies partly result from the lack of pharmacokinetic data in IBD patients.

Therefore, we investigated 6-mercaptopurine (6-MP) pharmacokinetics in a cohort of 30 IBD patients and found that 6-TGN and 6-MMPR concentrations showed large interpatient variability at all time points [22]. This was very similar to the results from pharmacokinetic studies in other patient populations [23, 24]. Steady state 6-TGN and 6-MMPR concentrations were reached after 4 weeks, suggesting a half-life of approximately 5 days for both metabolite groups [22]. This was previously demonstrated for 6-TGN, but not for 6-MMPR [25]. In our IBD-patient population on 6-MP (prescribed as a single oral 50 mg evening dose), steady state 6-TGN concentrations varied 7-fold and 6-MMPR concentrations reached an even larger 16-fold range [22].

Drug dose per kilogram bodyweight correlated neither with 6-TGN concentrations nor with 6-MMPR concentrations (figure 1)[22]. These data do not support the use of standard doses in mg/kg/day, as is commonly advised, because this results in unpredictable thiopurine metabolite concentrations. Metabolite concentrations that are too high may lead to drug induced toxicity, while subtherapeutic concentrations may cause inefficacy of thiopurine treatment.

Figure 1. Correlation between 6-MP dose and metabolite concentrations. 6-MP, 6-mercaptopurine; 6-TGN, 6-thioguaninenucleotides; 6-MMPR, 6-methylmercaptopurine ribonucleotides; RBC, red blood cell.

The obvious next question is what therapeutic lower and upper limits to use for both 6-TGN and 6-MMPR. In one of the first clinical studies in which thiopurine metabolites were assayed, a correlation was found between 6-TGN levels and therapeutic efficacy on one hand and between 6-MMPR levels and hepatotoxicity on the other [10]. In a subsequent study specific metabolite target levels were proposed: the frequency of therapeutic response increased at 6-TGN levels above 235 picomoles/ 8×10^8 RBC and hepatotoxicity correlated with 6-MMPR levels above 5700 picomoles/ 8×10^8 RBC. In a subpopulation with leukopenia a mean 6-TGN level of 490 picomoles/ 8×10^8 RBC was measured [11].

Keeping this therapeutic window in mind, we prospectively studied the correlations between these metabolite levels and both the occurrence of adverse events and efficacy. In our study, only one third of patients had steady state 6-TGN levels in the proposed therapeutic window of 235-490 picomoles/ 8×10^8 RBC. Half of the remaining patients developed steady state concentrations below 235 picomoles/ 8×10^8 RBC and above 490 picomoles/ 8×10^8 RBC respectively. Three patients had 6-MMPR concentrations above 5700 picomoles/ 8×10^8 RBC [22].

We then evaluated whether patients with metabolite levels outside the therapeutic window experienced more toxicity or reduced efficacy compared to patients with metabolite levels within the proposed window. Four patients (13%) developed leukopenia and all had a 6-TGN concentration above 490 picomoles/ 8×10^8 RBC. Moreover, all leukopenic patients had 6-TGN concentrations above 300 picomoles/ 8×10^8 RBC in the first week. Hence, a dose reduction would be recommended if a 6-TGN concentration above 300 picomoles/ 8×10^8 RBC is measured after 1 week of azathioprine (AZA) or 6-MP treatment [22].

Overall, 6-MMPR concentrations did not correlate with hepatotoxicity in our study in

Figure 2. Metabolite levels in an ultra-methylator; 6-MP, 6-mercaptopurine; 6-TGN, 6-thioguanine nucleotides; 6-MMPR, 6-methylmercaptopurineribonucleotides; RBC, red blood cell.

contrast to the findings of Dubinsky and colleagues [11]. However, in the subgroup of so-called ultra-methylators, patients with a preferential overproduction of 6-MMPR when given AZA or 6-MP, hepatotoxicity can be explained by elevated 6-MMPR levels for most part [26]. In a series of case reports, we described such a patient with preferential overproduction of 6-MMPR and hepatotoxicity after dose escalation because of suboptimal 6-TGN levels (figure 2) [27].

Three patients (10%) developed pancreatitis in our study. 6-MMPR concentrations did not correlate with the occurrence of pancreatitis, although one patient developed high 6-MMPR levels and pancreatitis simultaneously 6 weeks after start of treatment (8224 picomoles/8x10⁸ RBC) [22]. Pancreatitis is often considered to be an idiosyncratic reaction to thiopurines, but sometimes 6-MMPR concentrations are grossly elevated in patients with pancreatic events [10, 11]. Therefore, until now the role of 6-MMPR in the pathogenesis of pancreatitis remains uncertain.

Furthermore, extremely high 6-MMPR levels may also cause myelotoxicity. In the same series of case reports as mentioned before, we describe a patient with an extremely rare profile never described before [27]. This patient developed a reversible pancytopenia 7 weeks after start of 6-MP (1.7 mg/kg/day) and appeared to be an extraordinary ultra-methylator with a 6-TGN level of 126 picomoles/8x10⁸ RBC and a 6-MMPR level as high as 57000 picomoles/8x10⁸ RBC. This case points out that 6-TGN are not the only thiopurine metabolites to be reckoned with in the pathogenesis of myelotoxicity.

However our study was not powered to evaluate efficacy, we found efficacy to be associated with a 6-TGN concentration above the proposed lower therapeutic limit of 235 picomoles/8x10⁸ RBC in accordance with the results of Dubinsky and colleagues [11]. Ten patients had active IBD at baseline and 6 of them achieved clinical remission at week 8; 5 of the 6 patients (83%) had 6-TGN concentrations above 235 picomoles/8x10⁸ RBC and 3 of the 4 patients (75%) with no clinical improvement had concentra-

tions below the proposed threshold [22]. In addition, a 6-MMPR/6-TGN ratio less than 11 correlated with drug efficacy, as was also previously suggested by others: 4 of the 6 patients (67%) with a ratio under 11 achieved clinical remission within the study period, whereas 3 of the 4 patients (75%) with a ratio over 11 failed to do so [15, 22]. Non-compliance is another well-known problem, often seen in chronic patients in general and IBD patients in particular. In IBD patients treated with thiopurines, non-compliance will result in suboptimal thiopurine metabolite concentrations and TDM is the only method to reveal this phenomenon. By employing TDM during thiopurine treatment, we exposed several non-compliant IBD patients not daring to tell or to admit to their gastroenterologist that they were not taking the prescribed medication [27, 28]. Possible explanations for measured steady state thiopurine metabolite concentrations in IBD patients are summarized in table 1.

6-TGN concentrations between 250-500 picomoles/ 8×10^8 RBC and 6-MMPR concentrations below 5700 picomoles/ 8×10^8 RBC are considered to be the optimal metabolite levels. 6-TGN concentrations below 250 picomoles/ 8×10^8 RBC in combination with 6-MMPR concentrations below 5700 picomoles/ 8×10^8 RBC indicate non-compliance in case of very low or zero metabolite levels or under-dosing in case of low metabolite levels. If 6-TGN concentrations are below 250 picomoles/ 8×10^8 RBC combined with 6-MMPR concentrations above 5700 picomoles/ 8×10^8 RBC, patients are considered ultra-methylators with an increased risk of developing hepatotoxicity or myelotoxicity in extreme cases. On the contrary, when 6-TGN concentrations above 500 picomoles/ 8×10^8 RBC in combination with 6-MMPR concentrations below 5700 picomoles/ 8×10^8 RBC are measured, patients are considered poor or intermediate methylators with an increased risk on myelotoxicity.

In conclusion, TDM seems to be an attractive approach in fine-tuning thiopurine ther-

Table 1. Possible utilization of metabolite measurement in inflammatory bowel disease patients treated with azathioprine or 6-mercaptopurine

Explanation	6-TGN level (in pmol/ 8×10^8 RBC)	6-MMPR level (in pmol/ 8×10^8 RBC)	6-MMPR/6-TGN ratio
therapeutic target achieved	250-500	<5700	5-25
non-compliance	<<250	<<5700	5-25
under-dosing	<250	<5700	5-25
potential myelotoxicity (absent TPMT activity)	>>500	<<5700	0
possible myelotoxicity (low TPMT activity)	>500	<5700	0
possible hepatotoxicity (high TPMT activity)	<250-500	>5700	30-100
potential myelotoxicity (very high TPMT activity)	<<250	>>5700	>>100

6-TGN, 6-thioguaninenucleotides; 6-MMPR, 6-methylmercaptopurine ribonucleotides; pmol, picomoles; RBC, red blood cell; TPMT, thiopurine S-methyltransferase.

apy of inflammatory bowel disease, but there is great need for an adequately powered prospective comparison of efficacy and toxicity between IBD patients with and without fine-tuning by means of metabolite measurements. Future study designs should also consider metabolite measurements week 1 and 4 to validate our findings [22].

Genotyping

Another strategy that may optimize thiopurine therapy of IBD is genotyping genes of key enzymes in their metabolism, such as thiopurine S-methyltransferase (TPMT) and inosine triphosphate pyrophosphatase (ITPA).

For TPMT, it has been demonstrated that patients with one or two mutant alleles have reduced enzyme activity, leading to elevated 6-TGN concentrations, resulting in an increased risk for developing bone marrow suppression [29-31]. However, the use of determining TPMT status, the exact moment when to perform genotyping and the clinical implications remain subject to discussion. Although TPMT genotyping seems to play a role in the prediction of early drug toxicity in patients who have not been previously exposed to AZA or 6-MP, the predictive value for myelotoxicity in patients established on thiopurines may in fact be limited [31-33].

Recently, also inosine triphosphate pyrophosphatase (ITPase) deficiency has been associated with thiopurine related toxicity. In a study with 62 IBD patients, it has been shown that ITPase deficient patients due to a C94A missense mutation are at risk for developing adverse events such as flu-like symptoms, rash and pancreatitis when treated with AZA or 6-MP [34]. However, these results were contradicted by two other studies with 73 and 41 IBD patients respectively [35, 36]. Therefore, studies with larger number of patients are needed to determine the true value of ITPA genotyping in IBD populations.

Consequently, we studied TPMT and ITPA allele frequencies and investigated the association between the resulting genotypes and thiopurine toxicity in 262 IBD patients established on AZA [37]. TPMT allele frequencies found in our cohort of IBD patients were very similar to those reported in other Caucasian populations [31, 38, 39]: 238 (90.8%) patients carried wild-type alleles, 17 (6.5%) and 6 (2.2%) proved heterozygous for TPMT*3A and TPMT*3C respectively and 1 (0.4%) patient was a homozygous TPMT *3A mutant. ITPA allele frequencies in our population however, were higher than reported in other populations [40, 41]: 173 (66.0%) patients carried wild-type alleles, 29 (11.1%) and 55 (21.0%) patients were heterozygous mutants for C94A and IVS2+A21C respectively and 1 (0.4%) and 4 (1.5%) homozygous mutants for C94A and IVS2+A21C respectively were identified. Five patients (1.9%) were compound heterozygotes for C94A and IVS2+A21C and 9 patients (3.4%) were compound heterozygotes for ITPA and TPMT mutant alleles [37].

Both ITPA C94A allele and TPMT mutant alleles were associated with AZA related leukopenia (leukocyte count $<3.0 \times 10^9/l$) with odds ratio's of 3.5 (CI95%: 1.1-11.0;

$p=0.046$) and 6.3 (CI05%: 2.1-18.6; $p=0.004$) respectively [37]. The only patient homozygous for TPMT (TPMT*3A) in our study has experienced severe leukopenia during the first 2 weeks of the treatment, necessitating medication withdrawal. Interestingly, the only homozygote for ITPA C94A found in the studied population did not develop leukopenia. Neither the ITPA IVS2+A21C allele, nor compound heterozygosity correlated with AZA related leukopenia. Also, no significant differences in ITPA and TPMT alleles could be observed between groups with and without hepatotoxicity.

It has been demonstrated indisputably that homozygous TPMT mutants, like the one patient in our cohort, will experience early and severe myelosuppression due to extremely high 6-TGN levels when treated with standard AZA dosages and TPMT genotyping prior to initiation is the only way of identifying those at risk [22, 31, 33]. For heterozygous TPMT mutants however, an increased risk of developing bone marrow suppression mainly in a subsequent stage exists, but the correlation with TPMT geno- or phenotype is less pronounced [29, 33]. TPMT heterozygosity is just one of many risk factors such as concurrent medication (drugs interfering with AZA metabolism or causing bone marrow suppression by their own) or intercurrent viral infections and will not necessarily lead to myelosuppression [22, 33].

Despite the fact that we demonstrated a significantly increased odds ratio for ITPA mutants to develop leukopenia, we think that ITPA genotyping is probably not of great clinical importance as the ITPA homozygous mutant did not develop leukopenia. Therefore, the hypothesis that ITPase deficiency causes AZA related toxicity by accumulating the proposed metabolite 6-thioinosine triphosphate (6-TITP) seems unlikely. Until now, no pharmacogenetic study on ITPase deficiency included 6-TITP concentration measurements. Therefore, future study designs should consider 6-TITP measurement to elucidate the pharmacological mechanism behind AZA related toxicity in ITPase deficient patients.

6-Thioguanine

The administration of 6-TG as such, an agent more directly leading to the formation of the active 6-TGN, is considered to be another promising strategy avoiding AZA or 6-MP related toxicity or enhancing efficacy. 6-TG has been available for decades for patients suffering from leukemia but its use was virtually abandoned by the arrival of other more effective regimens. The interest in 6-TG has been renewed after its use was successfully studied in a small cohort of IBD patients therapy-resistant to AZA or 6-MP (ultra-methylators) [26].

Also in patients with AZA- or 6-MP-intolerance, 6-TG was considered to be an attractive approach because theoretically the number of possible toxic metabolites formed is strongly reduced [42]. We therefore performed a short-term safety assessment with 32 AZA- or 6-MP-intolerant IBD patients and demonstrated that 81% was able to tolerate 6-TG subscribed in a daily dose of 20-40 mg in the first 8 weeks of treatment [43]. Six

patients discontinued 6-TG due to the occurrence of adverse events, the relationship between 6-TG and adverse events being distinct in half of cases [43].

No serious hepatotoxicity occurred in the observed period. Moreover, three patients with non-severe pancreatitis within the first 4 weeks of AZA or 6-MP treatment did not develop pancreatitis within the first 8 weeks of 6-TG treatment [43]. This may indicate a role for 6-MMPR in the development in the pathogenesis of pancreatitis in at least a part of these patients because 6-MMPR levels were substantial on AZA and 6-MP while undetectable on 6-TG, but then again this relationship is disputable.

Mean 6-TGN concentrations were 937 and 1621 picomoles/ 8×10^8 RBC for 20 and 40 mg 6-TG daily respectively and considerably higher compared to 6-TGN levels on AZA or 6-MP [43]. Comparable results were found in similar short-term studies with AZA- or 6-MP-intolerant IBD populations that were given 6-TG in daily doses ranging from 10-40 mg [44, 45].

Despite the fact that 6-TGN concentrations were well above the proposed therapeutic upper limit of approximately 500 picomoles/ 8×10^8 RBC in patients using AZA or 6-MP, no myelotoxicity occurred in our cohort of patients [43]. Others confirmed this finding and several explanations were formulated [26, 45]. It was speculated that 6-TG derived 6-TGN are biochemically different from 6-MP derived 6-TGN [26]. Lancaster and colleagues offered a more convincing explanation: despite the accumulation of significantly higher erythrocyte 6-TGN concentrations for 6-TG compared with 6-MP, the accumulation of leukocyte 6-TGN in patients taking 6-TG was similar to the range of leukocyte 6-TGN in patients taking 6-MP [46]. In other words, when correlating intracellular 6-TGN to efficacy and myelotoxicity, RBC 6-TGN concentrations will be higher for patients taking 6-TG than in those taking 6-MP, whereas this is not the case in the putative target-tissue, the leukocytes. In addition to this explanation, 6-methylmercaptapurine ribonucleotides (6-MMPR), which arise from 6-MP but not from 6-TG, are believed to contribute to the immunosuppressive and myelotoxic effects [47].

Encouraged by these promising short-term data we then studied the tolerability and safety profile of a low-dose (0.3-0.4 mg/kg/day) maintenance therapy with 6-TG in 95 AZA or 6-MP intolerant IBD patients over a treatment period of at least 1 year and found out that 79% was able to tolerate long-term treatment [48]. Twenty patients discontinued 6-TG due to the occurrence of 26 adverse events of which gastrointestinal complaints (31%), hepatotoxicity (15%) and general malaise (15%) were the most frequently occurring [48]. The mean 6-TGN concentrations measured in the 6-TG-intolerant cohort was 725 picomoles/ 8×10^8 RBC compared to 540 picomoles/ 8×10^8 RBC in the tolerant cohort, but this difference was not statistically significant [48].

While performing our study disturbing high frequencies of nodular regenerative hyperplasia (NRH) of the liver were reported in an IBD population treated with 6-TG by Dubinsky and colleagues: NRH occurred in 76% of patients undergoing biopsy after laboratory abnormalities [49, 50]. NRH is a serious complication also associated with AZA and 6-MP and a frequent cause of portal hypertension [51, 52]. Therefore we im-

mediately performed an abdominal ultrasonography in the 54% of patients already using 6-TG for 1 year and discovered only one patient with signs of portal hypertension indicated by an enlarged spleen [48]. Furthermore, mean steady state 6-TGN concentrations measured in Dubinsky's population (~ 1250 picomoles/ 8×10^8 RBC) were significantly higher than in our population (~ 570 picomoles/ 8×10^8 RBC) and 6-TG induced NRH may in fact be dependent of the reached 6-TGN concentrations. This hypothesis, though contradicted by Dubinsky's data [49], merits further exploration and because biochemistry and ultrasound outcomes probably underestimate the real incidence of 6-TG induced NRH as the golden standard is based on histology we already started a liver biopsy study in our 6-TG-treated cohort.

In our study 73% clinically benefited from 6-TG evaluated by a global physician score and the mean albumin level increased significantly [48]. However, we must be reserved in drawing firm conclusions as our retrospective study was not designed to study efficacy and no standard efficacy parameters as the Crohn's disease activity index (CDAI) or Truelove-Witts disease activity index (TWDAI) were used. Nevertheless, our findings are supported by others who demonstrated 6-TG efficacy in a 24-week prospective study with 37 patients with active CD: 35% achieved remission (CDAI < 150) and 57% achieved a response (decrease of > 70 points in CDAI) [53]. In a follow-up study, remission was maintained for one year in 88% of responders to 6-TG [54]. More prospective trials designed to study efficacy are needed in IBD populations before 6-TG can be advocated in these patients. Naturally, it is not ethical to perform these studies before more knowledge is gathered about the potential hepatotoxicity of 6-TG.

It is unclear whether the measurement of 6-TG derived 6-TGN, the main metabolites, should guide dosing. That is, therapeutic drug monitoring is suggested in AZA- or 6-MP-treated patients but similar pharmacokinetic studies with 6-TG derived 6-TGN are scarce [55]. In fact, very little is known of 6-TG pharmacokinetics in IBD patients, metabolite levels and their correlation with drug efficacy and toxicity and we therefore performed a prospective pharmacokinetic study in 28 IBD patients. We found that 6-TGN concentrations showed large interpatient variability, similar to pharmacokinetic data in leukemic populations on 6-TG [56, 57]. 6-TG derived 6-TGN concentrations reached steady state after 4 weeks, similar to AZA or 6-MP derived 6-TGN concentrations [22, 58]. At steady state there was a 5-fold range in steady state 6-TGN concentrations, which is substantial but less pronounced than the range in 6-TGN originating from AZA or 6-MP [22, 58]. Beforehand, we expected a lesser variation in steady state 6-TGN levels as 6-TGN derived from 6-TG are formed in one single step compared to multiple steps when originating from AZA and 6-MP. In addition to this, 6-TG is less affected

by TPMT and a poor substrate for xanthine oxidase (XO) when compared to 6-MP and genetic polymorphisms of these metabolizing enzymes are of less influence [57]. Despite this fact, Herrlinger and colleagues identified one patient with TPMT heterozygosity (TPMT*1S/*3A) with bone marrow toxicity on 6-TG after a dose escalation from 40 to 80 mg/day in a cohort of 26 CD patients [55]. In our cohort all genotyped

subjects possessed wild-type alleles, so the high variability in metabolite levels could not be explained by genetic polymorphisms. Unfortunately, in half number of patients with 6-TG-intolerance TPMT genotype was not assessed and conclusions about the contribution of pharmacogenetics can not be drawn.

Though we found a correlation between absolute drug dose in mg and steady state 6-TGN concentration in our short-term safety assessment, no correlation could be demonstrated between 6-TGN concentrations and relative drug dose in mg per kilogram bodyweight in our pharmacokinetic study (figure 3) [43, 58]. This can be explained by the difference in the defined unit for drug dose; a relative drug dose seems a more appropriate unit in case of thiopurines.

In contrast to AZA and 6-MP, for 6-TG no correlation was found between 6-TGN concentrations and neither drug toxicity nor efficacy [58]. Similar results were reported by others [55]. Also, the proposed therapeutic window for AZA and 6-MP seems not applicable to 6-TG. Dubinsky and colleagues suggested a therapeutic lower limit of 1300 picomoles/ 8×10^8 RBC in IBD patients on 6-TG [44], but all our patients in whom remission was achieved in the observed period had 6-TGN concentrations below the proposed value. In another study, the median 6-TGN concentration measured was 648 picomoles/ 8×10^8 RBC and 42% of patients achieved remission suggesting that lower therapeutic target 6-TGN levels may be as effective [45]. All patients who achieved remission in our study had 6-TGN concentrations above this value but then again, so did the majority of patients who still experienced active disease [58]. Despite the fact that we could not demonstrated a significant relationship between 6-TGN levels on one hand and efficacy and toxicity on the other, we still believe that TDM may be a helpful tool in dosing 6-TG. Extremely high 6-TGN concentrations can be prevented, thereby

Figure 3. Correlation between 6-TG dose and metabolite concentrations. 6-TG, 6-thioguanine; 6-TGN, 6-thioguaninenucleotides; RBC, red blood cell.

myelotoxicity and possibly NRH, and compliance can be monitored.

Conclusions

TPMT genotyping and TDM are useful instruments for individualizing thiopurine pharmacotherapy of IBD. TPMT genotyping before initiation of treatment prevents early and severe myelosuppression by identifying homozygous mutants. Furthermore, in case of TPMT heterozygosity, safety measures such as precautionary dose reductions can be taken. Subsequently, TDM can guide thiopurine dosing to optimal metabolite levels herewith reducing the risk of drug related toxicity and increasing the chance on drug efficacy. Furthermore, TDM is useful whenever non-compliance is suspected. ITPA genotyping may be helpful in case of inexplicable myelotoxicity. At present, it is clear however that neither TDM nor TPMT or ITPA genotyping will replace or exclude frequent leukocyte monitoring in the screening for possible myelotoxicity. In case of AZA- or 6-MP-intolerance, 6-TG seems a promising alternative. However, in the near future more knowledge needs to be gathered about its potential hepatotoxicity before it can be advocated or banned.

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Samenvatting

Inleiding

De thiopurines azathioprine (AZA) en 6-mercaptopurine (6-MP) worden veelvuldig toegepast bij de behandeling van inflammatoire darmziekten (IBD). Deze immuno-suppressiva worden ingezet voor zowel het induceren van remissie als de onderhouds-behandeling van de ziekte van Crohn (CD) en colitis ulcerosa (UC) indien er onvoldoende respons is op mesalazine (-analogen) of wanneer er sprake is van corticosteroïdresistentie of -afhankelijkheid. In de klinische praktijk blijkt echter dat een derde van alle IBD-patiënten die thiopurines gebruiken, geen baat heeft bij deze middelen en dat een vijfde van diezelfde groep patiënten ze als gevolg van ernstige geneesmiddelgerelateerde bijwerkingen niet verdraagt. Deze omvangrijke groep van thiopurineresistente en -intolerante patiënten was tot voor kort overgeleverd aan alternatieve therapieën of chirurgie. De afgelopen decennia is de kennis over thiopurine farmacologie en farmacogenetica fors toegenomen, hetgeen heeft geleid tot de ontwikkeling van nieuwe strategieën voor optimalisatie van de farmacotherapie van IBD. Deze strategieën omvatten therapeutisch drug monitoring (TDM) van thiopurine-metabolieten, het genotypen van cruciale enzymen in het metabolisme van de thiopurines en de toepassing van 6-thioguanine (6-TG) als zodanig. Het onderzoek dat in het kader van dit proefschrift is uitgevoerd heeft bijgedragen aan de ontwikkeling van deze strategieën.

Algemene introductie

Deel I is een inleidend overzicht. **Hoofdstuk 1** is een ‘state of the art’ review over de toepassing van thiopurines bij IBD. Daarbij wordt ingegaan op hun effectiviteit en toxiciteit, farmacologie, farmacogenetische aspecten, interacties met andere geneesmiddelen en worden nieuwe strategieën om de thiopurines beter en veiliger te kunnen inzetten besproken. In **hoofdstuk 2** wordt dieper ingegaan op de toxiciteit van de thiopurines en manieren om de kans hierop te minimaliseren.

AZA en 6-MP zijn effectieve geneesmiddelen voor zowel de inductie van remissie als het behoud daarvan bij CD en UC, waarbij ze bij CD net wat effectiever lijken te zijn. Daarnaast voorkomen beide middelen exacerbaties van CD na een chirurgische ingreep en zijn ze in combinatie met andere immunosuppressiva effectief gebleken bij perianale fistels. Een bijkomend voordeel van het inzetten van de thiopurines is bovendien hun corticosteroïdsparing effect, waardoor de ernst en het aantal corticosteroïdgerelateerde bijwerkingen sterk kan worden teruggedrongen.

De bijwerkingen van de thiopurines kunnen worden onderverdeeld in dosisgerelateerde, farmacologisch goed verklaarbare bijwerkingen (type A) en dosisonafhan-

kelijke overgevoelighedsreacties (type B). Type A bijwerkingen treden dikwijls in een later stadium op, worden grotendeels in verband gebracht met de vorming van potentieel toxische metabolieten en omvatten onder andere algehele malaise en misselijkheid, infectieuze complicaties, hepatitis en beenmergdepressie. Type B bijwerkingen daarentegen treden veelal binnen 3 tot 4 weken na start op en resulteren in immunogelerelateerde symptomen zoals koorts, huiduitslag en gewrichtspijn.

Thiopurines zijn prodrugs die voor hun immunosuppressieve werking moeten worden omgezet in actieve metabolieten. AZA wordt snel en non-enzymatisch omgezet in 6-MP dat vervolgens intracellulair via een complex metabolisme door diverse enzymen wordt omgezet in onder andere de 6-thioguaninenucleotiden (6-TGN) en de 6-methylmercaptopurine ribonucleotiden (6-MMPR). De farmacologische werking van de thiopurines is slechts ten dele opgehelderd, maar wordt voornamelijk grotendeels toegeschreven aan de 6-TGN. Deze metabolieten vertonen sterke gelijkenis met de purinebase guanine en worden als zodanig ingebouwd in leukocyt-DNA, resulterend in cytotoxiciteit en immunosuppressie. Daarnaast remt één van de 6-TGN, 6-thioguanintrifosfaat (6-TGTP), Rac-1, hetgeen uiteindelijk apoptose van de T-cel tot gevolg heeft. Tenslotte dragen ook de 6-MMPR bij aan het immunosuppressieve effect door remming van de *de novo* purinesynthese.

Belangrijke enzymen in het thiopurinemetabolisme zijn thiopurine S-methyltransferase (TPMT), xanthineoxidase (XO), hypofosforibosyltransferase (HPRT) en inosinetrifosfaatpyrofosfatase (ITPase). Interindividuele variaties in therapeutische respons en toxiciteit kunnen deels worden verklaard door de variabele vorming van actieve metabolieten als gevolg van genetische polymorfismen in de genen die coderen voor deze enzymen. Een van de meest onderzochte genetische polymorfismen is dat van TPMT, waarvoor duidelijk is aangetoond dat patiënten met mutant allelen een verhoogd risico hebben op het ontwikkelen van leukopenie.

Geneesmiddelen die het thiopurine metabolisme beïnvloeden kunnen interacties geven met thiopurines. Zo wordt TPMT activiteit geremd door de aminosalicylaten (5-ASA), acetylsalicylzuur en furosemide en inhibeert allopurinol XO. In geval van laatst genoemde interactie is een reductie van de thiopurinedosering een absolute noodzaak. Voorzichtigheid is geboden bij het gelijktijdig toedienen van andere immunosuppressiva vanwege een potentieel synergistisch effect.

In de laatste jaren zijn diverse strategieën ontwikkeld om de farmacotherapie van IBD met thiopurines te optimaliseren: het meten van metabolietpiegels (TDM), geno- of fenotypering van cruciale enzymen in thiopurinemetabolisme en het toepassen van 6-TG als zodanig.

In een van de eerste studies op het gebied van TDM werd aangetoond dat 6-TGN en 6-MMPR spiegels correleren met respectievelijk effectiviteit en toxiciteit. In een andere studie werden vervolgens referentiewaarden voorgesteld: de kans op therapeutisch effect nam toe met een 6-TGN spiegel $> 235 \text{ picomol}/8 \times 10^8$ rode bloedcel (RBC), terwijl hepatotoxiciteit werd geassocieerd met een 6-MMPR spiegel $> 5700 \text{ picomol}/8 \times 10^8$ RBC. In een subpopulatie met leukopenie werd een 6-TGN spiegel van 490

picomol/ 8×10^8 RBC gemeten. Sindsdien zijn er een groot aantal publicaties verschenen over spiegelmetingen van metabolieten met nogal tegenstrijdige resultaten. De discussie over de klinische toepasbaarheid van TDM van thiopurines bij IBD populaties wordt mede gevoed door gebrek aan farmacokinetische data.

Genotyperen is een andere veelbelovende manier om de kans op toxiciteit van thiopurines te verminderen. Patiënten met één of twee mutant TPMT-allelen hebben TPMT met een verlaagde enzymactiviteit, resulterend in hogere 6-TGN spiegels en een verhoogd risico op het ontwikkelen van leukopenie. Er wordt daarom ook wel geadviseerd heterozygoot mutanten 50% van de normale thiopurine startdosering te geven en homozygoot mutanten maximaal 10% of helemaal geen thiopurine. Toch is het nog niet duidelijk of, en zo ja op welk moment, TPMT genotyperen klinisch relevant is, omdat er ook op dit gebied tegenstrijdige onderzoeksresultaten gepubliceerd zijn. Genotyperen lijkt zinvol vóór aanvang van thiopurinetherapie, maar de meerwaarde lijkt beperkt bij reeds ingestelde patiënten. Ook het ITPase-gen (ITPA) lijkt een interessante kandidaat voor genotypering. Recentelijk werd in een kleinschalige studie correlatie aangetoond tussen een ITPA C94A mutatie en AZA gerelateerde bijwerkingen zoals griepachtige symptomen, rash en pancreatitis. In een ander onderzoek konden deze resultaten echter niet worden gereproduceerd. Er is daarom behoefte aan onderzoek met een groot cohort van IBD-patiënten die thiopurines gebruiken, om de onduidelijkheden met betrekking tot het genotyperen van TPMT en ITPA op te helderen.

Weer een andere strategie om AZA of 6-MP gerelateerde toxiciteit te reduceren of het therapeutisch effect te verbeteren is het toepassen van 6-TG, een thiopurine die via één enkele stap wordt omgezet in de actieve 6-TGN en tot voor kort alleen gebruikt voor diverse vormen van leukemie. In een kleinschalige pilotstudie werd 6-TG succesvol ingezet bij zogenaamde ultramethyleerders, patiënten die op AZA of 6-MP ondanks dosisverhogingen geen adequate 6-TGN spiegels ontwikkelen, maar wel toxische 6-MMPR spiegels. Hoewel 6-TGN spiegels na 6-TG gebruik een factor 9 hoger waren vergeleken met 6-MP, ondervond geen enkele patiënt myelotoxiciteit. Theoretisch lijkt 6-TG ook een interessante optie in geval van AZA of 6-MP intolerantie, omdat het aantal potentieel toxische metabolieten sterk gereduceerd is. Voordat 6-TG buiten studieverband in de klinische praktijk kan worden ingezet bij IBD, moeten belangrijke onderwerpen als korte- en langetermijn veiligheid, effectiviteit en de betekenis van hoge 6-TGN spiegels gedurende langere tijd zorgvuldig worden bestudeerd.

Therapeutic drug monitoring

In **deel II** wordt onderzoek naar de klinische relevantie van metabolietspiegelmetingen gepresenteerd. In **hoofdstuk 3** wordt een studie beschreven waarin de farmacokinetiek van 6-MP prospectief is bestudeerd bij 30 IBD-patiënten. Metabolietspiegels werden bepaald 0, 1, 2, 4 en 8 weken na start van 50 mg 6-MP eenmaal daags 's avonds.

Primaire eindpunten van deze studie waren RBC 6-TGN en 6-MMPR spiegels. Secundaire eindpunten waren de correlatie van deze spiegels met respectievelijk 6-MP dosering (in mg/kg), TPMT genotype, hematologische-, hepatische- en pancreatische parameters. Een Crohn's disease activity index (CDAI) werd meegenomen als maat voor effectiviteit bij CD-patiënten, terwijl voor UC-patiënten een Truelove-Witts disease activity index (TWDAI) werd bepaald.

Steady state spiegels voor zowel 6-TGN als 6-MMPR werden bereikt na 4 weken, hetgeen een halfwaardetijd van ongeveer 5 dagen impliceert voor beide metabolieten. Voor 6-TGN was dit reeds bekend, voor 6-MMPR nog niet. Gemiddelde steady state spiegels waren 368 (CI95%:284-452) picomol/8x10⁸ RBC voor 6-TGN en 2837 (CI95%:2101-3537) picomol/8x10⁸ RBC voor 6-MMPR. Er werd een grote interindividuele variabiliteit in steady state metaboliet spiegels waargenomen: deze varieerden 7-voudig voor 6-TGN en zelfs 16-voudig voor 6-MMPR. 6-TGN en 6-MMPR spiegels correleerden noch met de 6-MP dosering (in mg/kg), noch met elkaar. In ons cohort werd een homozygoot (*3A) mutant geïdentificeerd en 4 heterozygoot (2 maal *1/*3A en 2 maal *1/*3C) TPMT mutanten. Er werd een correlatie gevonden tussen TPMT genotype en de 6-TGN spiegel en patiënten met TPMT mutant allelen hadden een relatief risico van 12,0 (CI95%:1,7-92,3) op het ontwikkelen van leukopenie. Vier patiënten ontwikkelden een leukopenie in de studieperiode, waarbij deze het snelst optrad (na 1 week) en het meest uitgesproken was bij de homozygoot TPMT mutant. Twee heterozygoot mutanten en een wild-type patiënt kregen eveneens leukopenie na respectievelijk 2, 4 en 8 weken. Alle patiënten bij wie op den duur leukopenie ontstond, hadden na 1 week reeds een 6-TGN spiegel van tenminste 300 picomol/8x10⁸ RBC. Drie patiënten ontwikkelden een pancreatitis en 1 patiënt ondervond hepatotoxiciteit van 6-MP, maar er werd geen correlatie gevonden met de 6-MMPR spiegel. Hoewel de studie hier niet voor ontworpen was, kon er een duidelijk verband worden aangetoond tussen 6-TGN spiegels enerzijds en effectiviteit anderzijds: 83% van de patiënten met actieve ziekte bij aanvang en remissie na 8 weken hadden 6-TGN spiegels boven de voorgestelde therapeutisch ondergrens van 235 picomol/8x10⁸ RBC, terwijl 75% van de patiënten die geen klinische progressie maakten 6-TGN spiegels onder deze waarde had. Deze studie toonde aan dat bij IBD-patiënten die 6-MP gebruiken, de spreiding in metaboliet spiegels groot is, hetgeen niet pleit voor het doseren in mg/kg zoals tot op de dag van vandaag gebruikelijk is. Daarnaast liet deze studie zien dat TPMT genotypering en vooral TDM een nuttig instrument kunnen zijn om de farmacotherapie van IBD met thiopurines te optimaliseren.

Hoofdstuk 4 is een bundeling van een vijftal case-reports die het nut dat TDM kan hebben ter begeleiding van thiopurinetherapie van IBD, duidelijk illustreren. Casus 1 laat een patiënt met actieve linkszijdige UC zien die geen aantoonbare metaboliet spiegels had en therapie-ontrouw bleek. De tweede casus beschrijft een ultramethyleerder die na een 6-MP dosisophoging van 50 naar 100 mg een hoge 6-MMPR spiegel (8686 picomol/8x10⁸ RBC) ontwikkelde en als gevolg daarvan levertoxiciteit. Na staken van 6-MP en overzetting op 6-TG daalden de 6-MMPR spiegels en normaliseerden de

leverenzymwaarden. De patiënt uit de derde casus had reeds na 1 week een verhoogde 6-TGN spiegel (425 picomol/ 8×10^8 RBC) en bleek na TPMT genotypering een heterozygoot mutant (*1/*3A). Na halvering van de 6-MP dosis tot 25 mg/dag was de steady state 6-TGN spiegel acceptabel (417 picomol/ 8×10^8 RBC). De patiënt uit de vierde casus had na 1 week 6-MP 50 mg een extreem hoge 6-TGN spiegel van 1284 picomol/ 8×10^8 RBC en belandde met ernstige leukopenie en sepsis op de intensive care. TPMT genotypering wees uit dat deze patiënt een TPMT homozygoot mutant (*3A/*3A) was. Casus 5 is een zeer zeldzame: deze patiënt ontwikkelde 7 weken na start van 6-MP als gevolg van een extreem hoge TPMT activiteit een torenhoge 6-MMPR spiegel van maar liefst 57000 picomol/ 8×10^8 RBC, hetgeen uiteindelijk resulteerde in een lang aanhoudende pancytopenie. 6-MMPR spiegels van dit caliber waren tot dan toe nog niet in de literatuur beschreven. Bovendien is het interessant dat deze spiegels niet in hepatotoxiciteit, doch myelotoxiciteit resulteerden.

Genotyperen

In **deel III** wordt de meerwaarde van genotyperen onderzocht. **Hoofdstuk 5** beschrijft een retrospectieve studie waarin 262 met AZA behandelde IBD-patiënten werden genotypeerd voor TPMT (*2/*3A/*3B/*3C) en ITPA (C94A/IVS2+A21C), waarna beide genotypes werden gecorreleerd met het optreden van leukopenie en hepatotoxiciteit.

Van de 262 patiënten bezaten 238 patiënten wild-type TPMT allelen (90,8%), waren er 23 heterozygoot mutant (8,8%) en er werd 1 homozygoot mutant (0,4%) geïdentificeerd. Voor ITPA C94A werden er 1 homozygoot (0,4%) en 29 heterozygoot mutant (11,2%) geïdentificeerd, voor ITPA IVS2+A21C waren dat er respectievelijk 4 (1,5%) en 55 (21,0%).

Leukopenie trad op bij 4,6% van de behandelde patiënten. Mutant allelfrequenties van ITPA C94A en TPMT waren significant hoger in het cohort met leukopenie vergeleken met de patiënten zonder leukopenie: 16,6% en 5,4% respectievelijk voor ITPA en 20,8% en 4,0% respectievelijk voor TPMT. Zowel ITPA C94A als TPMT mutaties voorspelden leukopenie met een odds ratio van 3,5 (CI95%: 1,1-11,0) voor ITPA en 6,3 (CI95%: 2,1-18,6) voor TPMT. ITPA IVS2+A21C genotype was niet geassocieerd met het optreden van leukopenie. Hepatotoxiciteit trad op bij 4,2% van de patiënten. Geen van de onderzochte genotypes was geassocieerd met het optreden van hepatotoxiciteit. Het is onomstotelijk bewezen dat homozygoot TPMT mutanten een vroege, ernstige leukopenie zullen ervaren indien ze worden behandeld met standaard AZA doseringen, zoals ook bij de homozygoot mutant uit ons IBD-cohort het geval bleek. TPMT geno- of fenotypering voor start van thiopurinetherapie is vooralsnog de enige manier om deze hoogrisico patiënten aan te wijzen. Hoewel ook bij heterozygoot TPMT mutanten een verhoogd risico op leukopenie bestaat, is de correlatie met TPMT genotype minder uitgesproken. TPMT heterozygositeit is slechts een van de vele risicofactoren,

waartoe ook co-medicatie (geneesmiddelen die interfereren met het thiopurinemetabolisme of zelf ook immunosuppressief werken) en virale infecties behoren. Daarnaast leidt het hebben van één TPMT mutant allel niet altijd tot het ontwikkelen van leukopenie.

Ondanks de aangetoonde correlatie met leukopenie, is de meerwaarde van ITPA genotypering beperkt; de enige homozygoot mutant voor ITPA C94A ontwikkelde namelijk geen leukopenie in de studieperiode. Deze bevinding ondersteunt dan ook niet de hypothese, dat ITPA geïnduceerde leukopenie een dosis- of metabolietspiegelgerelateerd fenomeen is. Periodieke hematologische labcontroles lijken vooralsnog toereikend om ITPA geïnduceerde leukopenie te voorkomen.

6-Thioguanine

Deel IV beschrijft een drietal studies over de toepassing van 6-TG bij IBD. In **hoofdstuk 6** wordt dieper ingegaan op de farmacokinetiek van 6-TG. In beschreven prospectieve farmacokinetische studie werden metabolietspiegels bepaald 0, 1, 2, 4 en 8 weken na start van 20 mg 6-TG eenmaal daags 's avonds bij 28 IBD-patiënten. Het primaire eindpunt van deze studie was de RBC 6-TGN spiegel, het secundaire eindpunt was de correlatie van deze spiegel met respectievelijk 6-TG dosering (in mg/kg), TPMT genotype, hematologische-, hepatische- en pancreatische parameters. Een Crohn's disease activity index (CDAI) werd meegenomen als maat voor effectiviteit bij CD-patiënten, voor UC-patiënten werd een Truelove-Witts disease activity index (TWDAL) bepaald.

Steady state 6-TGN spiegels werden bereikt na 4 weken, hetgeen ook voor van 6-TG afkomstige 6-TGN een halfwaardetijd van ongeveer 5 dagen impliceert. De gemiddelde steady state 6-TGN spiegel was 856 (CI95%:715-997) picomol/8x10⁸ RBC. Er was een grote interindividuele variabiliteit in metabolietspiegels op alle tijdstippen: deze varieerden 5-voudig. Dit is weliswaar een minder grote interindividuele variatie dan aangetoond bij AZA en 6-MP gebruikers, maar desalniettemin substantieel. Voorafgaand aan de studie werd een minder grote variatie in 6-TGN spiegels verwacht, omdat 6-TG in tegenstelling tot AZA en 6-MP slechts in één stap wordt omgezet in de actieve 6-TGN. 6-TGN spiegels correleerden niet met de 6-TG dosering (in mg/kg), hetgeen niet pleit voor doseren in mg/kg van 6-TG bij IBD. Hoewel de 6-TGN spiegels op 6-TG aanzienlijk hoger waren in vergelijking met AZA of 6-MP, ontwikkelde geen enkele patiënt uit ons cohort myelotoxiciteit. In een andere studie werd hiervoor een verklaring gevonden: hoewel 6-TGN spiegels na 6-TG gebruik hoger waren dan na 6-MP gebruik in de erythrocyt, was dit niet het geval in het gewenste doelweefsel, de leukocyt. Er trad noch hepatotoxiciteit, noch pancreatitis op in de studiepopulatie, hoewel één patiënt tijdelijk verhoogde amylase- en lipasewaarden had zonder klinische symptomen. Er kon dan ook geen correlatie worden aangetoond tussen 6-TGN spiegels enerzijds en myelotoxiciteit, hepatotoxiciteit of pancreatitis anderzijds. Vier patiënten (14%)

ondervonden bijwerkingen van dien aard dat 6-TG therapie gestaakt moest worden. De relatie tussen 6-TG en toxiciteit kon worden vastgesteld bij één patiënt en was op zijn minst waarschijnlijk bij 2 patiënten. Er werd geen verband gevonden tussen 6-TGN spiegels en de effectiviteit van de 6-TG bij IBD. Dit is in tegenspraak met de onlangs voorgestelde therapeutische ondergrens van $1300 \text{ picomol}/8 \times 10^8 \text{ RBC}$.

Deze studie toonde aan dat ook bij IBD-patiënten die 6-TG gebruiken, de spreiding in metabolietspiegels groot is, hetgeen niet pleit voor het doseren in mg/kg. Hoewel geen correlatie tussen metabolietspiegels en toxiciteit of effectiviteit kon worden aangetoond, lijkt het raadzaam om regulier 6-TGN spiegels te bepalen om extreem hoge waarden en de nog onbekende gevolgen daarvan te voorkomen, totdat meer veiligheidsdata beschikbaar zijn.

Hoofdstuk 7 beschrijft een open, prospectieve multicenter studie naar de kortetermijn veiligheid van 6-TG bij IBD. Tweeëndertig AZA of 6-MP intolerante IBD-patiënten werden behandeld met 6-TG in een dosering van 20 mg (n=19) of 40 mg (n=13). Veiligheidsdata werden verzameld tot 8 weken na start van 6-TG. Het primaire eindpunt was de tolerantie van 6-TG.

Zesentwintig (81%) van de 32 patiënten verdroegen 6-TG gedurende de eerste 8 weken, daar waar AZA of 6-MP in dezelfde periode niet verdragen werd. Zes patiënten (19%) staakten farmacotherapie met 6-TG door het optreden van bijwerkingen. Bij 3 van de 6 patiënten was het gelegde verband tussen 6-TG gebruik en opgetreden toxiciteit plausibel. De gemiddelde steady state 6-TGN spiegels waren significant hoger op een dosering van 40 mg/dag ($1621 \text{ picomol}/8 \times 10^8 \text{ RBC}$) dan op 20 mg/dag ($937 \text{ picomol}/8 \times 10^8 \text{ RBC}$). Ondanks de hoge 6-TGN spiegels ontwikkelde niemand myelotoxiciteit. Ook trad er geen klinisch relevante hepatotoxiciteit of pancreatitis op, zelfs niet bij de patiënten waarbij dit wel optrad na AZA of 6-MP gebruik.

Deze studie liet zien dat 6-TG een interessante optie lijkt in geval van AZA of 6-MP intolerantie omdat het merendeel van de patiënten deze thiopurine wel verdraagt. Deze resultaten moedigden dan ook aan tot verder, langduriger veiligheidsonderzoek naar de toepassing van 6-TG bij IBD.

De studie die hierop volgde is beschreven in **hoofdstuk 8**. In deze retrospectieve database-analyse, waarin het merendeel van de Nederlandse IBD-patiënten die tot dan toe werden behandeld met 6-TG was opgenomen, werd de tolerantie en het veiligheidsprofiel van 6-TG gedurende 1 jaar onderzocht bij 95 IBD-patiënten. De volgende gegevens werden in de database opgenomen: demografische gegevens, medicatiehistorie, indicatie voor 6-TG, eventuele thiopurine-intolerantie, co-medicatie, hematologische parameters, leverenzymwaarden en TPMT genotype.

Vijfzeventig (79%) van de patiënten uit de database verdroegen 6-TG gedurende tenminste 1 jaar. Twintig (21%) van de 95 patiënten staakten 6-TG gebruik als gevolg van bijwerkingen binnen 1 jaar na start. Tot deze bijwerkingen behoorden onder andere maagdarmklachten (31%), algehele malaise (15%), hepatotoxiciteit (15%), myelotoxiciteit (4%), pancreatitis (4%) en allergische reacties (4%). De gemiddelde absolute 6-TG startdosering was 24,6 mg/dag (range: 20-40 mg) terwijl de relatieve do-

sering 0,37 mg/kg/dag bedroeg. Hoewel de studie hier niet voor was ontworpen, kon de effectiviteit van 6-TG volgens de weliswaar redelijk subjectieve Global Physician Score worden bepaald bij 85% van de patiënten uit de database: 73% van de patiënten deed het beter dan daarvoor, 23% hetzelfde en 6% slechter. Bij 63% van de patiënten was een steady state 6-TGN spiegel bepaald en deze bedroeg gemiddeld 540 (range: 0-1404) picomol/8x10⁸ RBC. Bij 54% van de patiënten werd het TPMT genotype bepaald, onder wie 2 heterozygoot (*1/*3A en *1/*3C) mutanten welke beide intolerant bleken voor 6-TG. Een daarvan ontwikkelde leukopenie, terwijl de ander juist hepatotoxiciteit ondervond.

Ook deze studie toonde aan dat 6-TG een interessante optie is in geval van AZA of 6-MP intolerantie. Het merendeel van de patiënten verdroeg 6-TG gedurende tenminste 1 jaar en de bijwerkingen die optraden in de intolerante groep waren reversibel na staken van 6-TG.

Gedurende de uitvoering van deze studie werd door een andere onderzoeksgroep een hoge incidentie van nodulaire regeneratieve hyperplasie (NRH) van de lever gemeld bij IBD-patiënten die behandeld werden met 6-TG: NRH werd waargenomen bij 76% van alle patiënten met verhoogde leverenzymwaarden. NRH is een serieuze complicatie die ook in verband wordt gebracht met AZA en 6-MP en een frequente oorzaak is van portale hypertensie. Na publicatie van dit verontrustende bericht werd bij 54% van de patiënten uit onze database een echografie van het abdomen uitgevoerd en werden bij één patiënt aanwijzingen voor portale hypertensie gevonden aangeduid door een vergrote milt. Een echografie van het abdomen is echter niet de gouden standaard bij het diagnosticeren van 6-TG geïnduceerde NRH en onderschat wellicht de werkelijke incidentie. Gemeten steady state 6-TGN spiegels uit ons IBD-cohort waren ruim een factor 2 lager dan die gemeten in het cohort met de hoge NRH-incidentie hetgeen impliceert dat 6-TG geïnduceerde NRH mogelijk een spiegelgerelateerd fenomeen is. Deze hypothese verdient dan ook nader onderzoek gebaseerd op histologisch verkregen incidentiecijfers.

Slotbeschouwing

Tenslotte zijn in **deel V, hoofdstuk 9** alle conclusies en toekomstperspectieven nog eens op een rijtje gezet. TDM en TPMT genotypering zijn bruikbare instrumenten om de farmacotherapie van IBD met thiopurines te individualiseren. Indien TPMT genotypering plaatsvindt vóór start van behandeling, worden homozygoot mutanten geïdentificeerd en kan vroege, ernstige myelosuppressie worden voorkomen. Bovendien kunnen bij heterozygoot mutanten tijdig extra voorzorgsmaatregelen plaatsvinden zoals een dosisreductie en een frequentere bloedbeeldcontrole. Vervolgens kan TDM de thiopurinedosering optimaliseren op grond van metabolietspiegels, waardoor het risico op geneesmiddelgerelateerde toxiciteit wordt verminderd en de kans op effectiviteit vergroot. Daarnaast is TDM zinvol wanneer therapie-ontrouw wordt vermoed.

ITPA genotypering kan myelotoxiciteit zonder aanwijsbare oorzaak mogelijk helpen te verklaren. Toch is het duidelijk dat noch TDM, noch TPMT of ITPA genotypering frequente bloedbeeld controle ooit zal kunnen vervangen, omdat ook myelotoxiciteit optreedt bij patiënten met wild-type TPMT en ITPA genotype met metabolietpiegels in het voorgestelde referentiegebied. In geval van AZA of 6-MP intolerantie blijft 6-TG een interessante optie. In de nabije toekomst zal echter meer kennis moeten worden vergaard over de potentiële hepatotoxiciteit en effectiviteit van 6-TG voordat dit geneesmiddel buiten studieverband kan worden ingezet bij IBD of in zijn geheel moet worden verlaten.

Figuur 1. AZA, azathioprine; 6-MP, 6-mercaptopurine; 6-MMP, 6-methylmercaptopurine; 6-TUA, 6-thiourinezuur; 6-TIMP, 6-thioinosinemonofosfaat; 6-MTIMP, 6-methylthioinosinemonofosfaat; 6-MTIDP, 6-methylthioinosinedifosfaat; 6-MTITP, 6-methylthioinosinetrifosfaat; 6-MMPR, 6-methylmercaptopurine ribonucleotiden; 6-TIDP, 6-thioinosinedifosfaat; 6-TITP, 6-thioinosinetrifosfaat; 6-TXMP, 6-thioxanthosinemonofosfaat; 6-TGMP, 6-thioguaninemonofosfaat; 6-TGDP, 6-thioguaninedifosfaat; 6-TGTP, 6-thioguaninetrifosfaat; 6-TGN; 6-thioguaninenucleotiden; 6-MTGMP, 6-methylthioguaninemonofosfaat; 6-TG, 6-thioguanine; 6-MTG, 6-methylthioguanine; XO, xanthine oxidase; TPMT, thiopurine S-methyltransferase; ITPase, inosinetrifosfaatpyrofosfatase; HPRT, hypoxanthinefosforibosyltransferase; IMPD, inosinemonofosfaatdehydrogenase; GMPS, guanosinemonofosfaatsynthetase; MPK, monofosfaatkinase; DPK, difosfaatkinase.

Nawoord

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Luc

Curriculum vitae

Luc Derijks werd geboren op 7 december 1973 in het Noord-Brabantse Wintelre, gemeente Vessem. In 1992 behaalde hij het diploma Gymnasium β aan het Van Maerlantlyceum te Eindhoven, waarna hij begon met de studie Farmacie aan de Universiteit Utrecht. Het apothekersdiploma werd behaald in oktober 1998.

Vervolgens was hij ruim een jaar in dienst als projectapotheker bij de Stichting Ziekenhuisapothek en Laboratorium Venray. In 2000 werd begonnen met de opleiding tot ziekenhuisapotheker in het Maaslandziekenhuis te Sittard. Het in het kader van deze opleiding uitgevoerde registratieonderzoek vormde de basis voor dit proefschrift.

Na zijn registratie als ziekenhuisapotheker in 2003 werd gestart met de opleiding tot klinisch farmacoloog, die begin 2004 werd afgerond. Sinds 2004 is hij als stafid verbonden aan de apotheek van Máxima Medisch Centrum te Veldhoven.

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