The effect of allopurinol and low-dose thiopurine combination therapy on the activity of three pivotal thiopurine metabolizing enzymes: Results from a prospective pharmacological study

M.L. Seinen a,⁎, D.P. van Asseldonk a, N.K.H. de Boer a, N. Losekoot b, K. Smid b, C.J.J. Mulder a, G. Bouma a, G.J. Peters b, A.A. van Bodegraven a

a Department of Gastroenterology and Hepatology, VU University Medical Center, Amsterdam, The Netherlands
b Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands

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Abstract

Introduction: Thiopurine therapy is often discontinued in inflammatory bowel disease (IBD) patients. The xanthine oxidase (XO) inhibitor allopurinol has previously shown to enhance thiopurine efficacy and to prevent adverse reactions, the mechanism of this beneficial interaction is not completely clarified. The aim of this study is to observe possible effects of allopurinol and low-dose thiopurine combination therapy on the activity of three pivotal thiopurine metabolizing enzymes.

Methods: A prospective study of IBD patients failing thiopurine therapy due to a skewed thiopurine metabolism was performed. Patients were treated with allopurinol and azathioprine or mercaptopurine. Xanthine oxidase, hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and thiopurine S-methyl transferase (TPMT) activities, and thiopurine metabolites concentrations were measured during thiopurine monotherapy, and after 4 and 12 weeks of combination therapy.

Results: Of fifteen IBD patients, XO activity decreased from 0.18 (IQR 0.08–0.3) during thiopurine monotherapy to 0.14 (IQR 0.06–0.2) and 0.11 (IQR 0.06–0.2; p=0.008) mU/hour/ml at 4 and 12 weeks, respectively. HGPRT activity increased from 150 (IQR 114–176) to 180 (IQR 135–213) and 204 nmol/(h×mg protein) (IQR 173–213; p=0.013). TPMT activity seemed not to be affected. 6-Thioguanine nucleotide concentrations increased from 138 (IQR 119–188) to 235 (223–304) and to 265 pmol/8×10^8 (IQR 188–344), whereas 6-methyl mercaptopurine ribonucleotides concentrations decreased from 13230 (IQR 7130–17420) to 690 (IQR 378–1325) and 540 (IQR 240–790) pmol/8×10^8 at 4 and 12 weeks of combination therapy (both p<0.001).

⁎ Corresponding author at: Department of Gastroenterology and Hepatology, VU University Medical Center, PO Box 7057, 1007 MB, Amsterdam, The Netherlands. Tel.: +31 20 4440613; fax: +31 20 4440554.
E-mail address: ml.seinen@vumc.nl (M.L. Seinen).

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**Conclusion:** Allopurinol and thiopurine combination-therapy seems to increase HGPRT and decrease XO activity in IBD patients, which at least in part may explain the observed changes in thiopurine metabolite concentrations.

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1. Introduction

The immunomodulating pro-drugs azathioprine (AZA) and mercaptopurine (MP) are commonly used in the treatment of inflammatory bowel disease (IBD), as these conventional thiopurines are recommended in most IBD-guidelines as first line immunosuppressive maintenance treatment. Nevertheless, in daily practice up to half of IBD patients discontinue this therapy within 2 years. Treatment withdrawal is mostly due to the development of adverse drug reactions or therapy resistance, allegedly related to an aberrant metabolism, which seems theoretically disadvantageous.1,2

Several enzymes, of which the activities are partly pharmacogenetically determined, are crucial in the complex metabolism of thiopurines.3 Among these enzymes are: hypoxanthine-guanine phosphoribosyl transferase (HGPRT), xanthine oxidase (XO) and thiopurine S-methyl transferase (TPMT). Following conversion of AZA into 6MP, the first step in metabolism of 6MP to the pharmacologically active 6-thioguanine nucleotides (6-TGN) is driven by HGPRT. However, 6MP can also be oxidized by xanthine oxidase (XO) into inactive 6-thiouric acid (6TUA) or methylated by thiopurine S-methyl transferase (TPMT) either directly into 6-methyl mercaptopurine or as a nucleotide into 6-methyl mercaptopurine ribonucleotides. These methylated products together have been named 6-MMPR (Fig. 1).4 Patients with a skewed metabolism produce high red blood cell (RBC) 6-MMPR concentrations at the cost of 6-TGN formation. High 6-MMPR concentrations are associated with toxicity, in particular hepatotoxicity but even with myelotoxicity, whereas 6-TGN concentrations above a certain cut-off (>235 mmol/8 × 10^8 RBC) are associated with therapeutic efficacy.5–8 However, prospective studies have not be able to demonstrate that 6-TGN-guided dosing is superior to

![Scheme of the thiopurine metabolism](image)

**Figure 1** Following conversion of azathioprine (AZA) into 6-mercaptopurine (6MP), the first step in bioactivation of 6MP is mediated by hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and yields 6-thioinosine monophosphate (6TIMP). However, 6MP can also be oxidized by xanthine oxidase (XO) into inactive 6-thiouric acid (6TUA), or methylated by thiopurine S-methyl transferase (TPMT) into 6-methyl mercaptopurine (6MMP). Moreover, 6MP can be inactivated by aldehyde oxidase (AOX), into 2-hydroxy-6-mercaptopurine (2OHMP). 6-Thioinosine monophosphate (6TIMP) can further be metabolized in three different ways. First, 6TIMP is a substrate for TPMT, which results in the formation of 6-methyl mercaptopurine ribonucleotides (6-MMPR). These 6-MMPR include 6-methyl thioinosine monophosphate (6meTIMP), 6-methyl thioinosine diphosphate (6meTIDP) and 6-methyl thioinosine triphosphate (6meTITP). Second, 6TIMP can be phosphorylated via 6-thioinosine diphosphate (6TIDP) to 6-thioinosine triphosphate (6TITP), which in turn can be converted back to 6TIMP by inosine triphosphate pyrophosphohydrolase (ITPase). Third, inosine-5-monophosphate dehydrogenase (IMPDH) converts 6TIMP into 6-thioxanthosine monophosphate (6TXMP). 6-Thioxanthosine monophosphate (6TXMP), then, is converted by guanosine monophosphate synthetase (GMPS) into 6-thioguanine monophosphate (6TGMP), which in turn is phosphorylated by kinases to 6-thioguanine diphosphate (6TGD) and 6-thioguanine triphosphate (6TGTP). Together these nucleotides form 6-TGN.
standard weight-based dosing, partly due to lack of proper pharmacodynamic tests. The classical XO inhibitor allopurinol, usually prescribed for the treatment of gout, can enhance the efficacy of thiopurine therapy in renal transplant and IBD patients. The enhanced efficacy is assumed to be due to an increase of 6-TGN concentrations. As severe leukopenia may occur with high 6-TGN concentrations, thiopurine dosages need to be reduced to approximately 25% of their original weight-based dose during combination therapy. Sparrow and colleagues not only showed that upon allopurinol combination therapy 6-TGN concentrations increase, but also that 6-MMPR concentrations decrease. Moreover, 6-MMPR associated liver test abnormalities ameliorated during combination therapy. Recently, combination therapy of allopurinol and low-dose thiopurine in IBD patients was also shown to prevent non-hepatic adverse events that had occurred during standard dosed thiopurine monotherapy. Moreover, long-term treatment with this combination is effective and well-tolerated in IBD patients. The pharmacokinetic explanation of the substantial increase in 6-TGN and decrease in 6-MMPR concentrations upon combination therapy is not completely clarified, but may be explained by alterations in the activities of XO, TPMT and HGPRT. The aim of the present study was to observe possible effects of allopurinol and low-dose thiopurine combination therapy on XO, TPMT and HGPRT activities in IBD patients who previously failed thiopurine monotherapy, due to a skewed thiopurine metabolism.

2. Materials and methods

2.1. Study design

From April 2010 through January 2011, we performed a single centre uncontrolled prospective study at the Gastroenterology and Hepatology department of the VU University Medical Center in Amsterdam, The Netherlands. The study was carried out in accordance with the 2008 declaration of Helsinki. All patients were treated with 100 mg allopurinol once daily in addition to low-dosed (25–33% of normal weight-based dose) AZA or 6MP. Patients were monitored for 12 weeks.

2.2. Population

In this single centre study, all consecutive IBD patients between 18 and 70 years of age who discontinued AZA or 6MP therapy and displayed a skewed thiopurine metabolism were eligible for this study. Thiopurines were discontinued due to adverse reactions (including liver test abnormalities), or therapy resistance. Patients were consecutively recruited at the Outpatient Clinic. Therapy resistance was defined as on-going disease activity despite adequate dose escalation or steroid dependency for more than 6 months. All patients had a skewed thiopurine metabolism, arbitrarily defined as a 6-MMPR/6-TGN ratio above 20 during normal, bodyweight-based dosed conventional thiopurine therapy (1.0–1.5 mg/kg for 6MP and 2.0–2.5 mg/kg for AZA). Diagnosis of IBD was ascertained by standard clinical, radiological, histological and endoscopical criteria. Thiopurines were prescribed according to a step-up approach as recommended in current IBD guidelines.

2.3. Patient characteristics

The following patient characteristics were collected: gender, body mass index (BMI), type of IBD (Crohn’s disease (CD) or ulcerative colitis (UC), including Montreal classification), smoking history, age and duration of IBD. As small bowel surgery may interfere with absorption of thiopurine therapy, data regarding previous intestinal surgery was documented. Treatment characteristics included type of thiopurine, dosage during monotherapy and combination therapy, concomitant medications and reason for initiating combination therapy. In addition, adverse reactions during combination therapy were documented.

2.4. Evaluation of haematological and liver toxicity

For safety reasons haematological and liver test variables were monitored during thiopurine monotherapy, immediately prior to (week 0) and at weeks 1, 2, 4, 6, 8, and 12 of combination therapy. Blood tests included leukocyte count (WBC), haemoglobin (Hb), hematocrit (Ht), erythrocyte count, mean corpuscular volume (MCV), platelet count, C-reactive protein (CRP), bilirubin, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (AP), and γ-glutamyltranspeptidase (γ-GT) concentrations. Myelosuppression was defined as a WBC count ≤ 3.5×10^9/L. Liver test abnormalities were classified according to the Common Terminology Criteria for Adverse Events version 3.0. Grade 1 is defined as liver test values between the upper limit of normal (ULN) and 2.5×ULN, grade 2 as 2.5 to 5.0×ULN, and grade 3 as 5.0× to 20×ULN.

2.5. Thiopurine metabolites

During thiopurine monotherapy, immediately prior to and at weeks 4 and 12 of combination therapy, 6-TGN and 6-MMPR concentrations were measured in RBC by a high performance liquid chromatography (HPLC) assay according to a slightly modified version of the previously described method of Dervieux and Bouli. Measured 6-TGN concentrations by this Dervieux method were divided by 2.6 to make them comparable with those that would have been observed if using the method of Lennard and Singleton.

2.6. Enzyme assays

Activities of HGPRT, TPMT and XO were determined during thiopurine monotherapy, prior to and at weeks 4 and 12 of combination therapy. Blood samples were immediately handled and stored at −20 degrees Celsius after isolation of RBC and plasma. All enzyme activities were measured in a 37 degrees Celsius shaking water bath, at saturating substrate concentration and hence reflect a Vmax. The protein concentrations were measured with the Bradford assay. From this series, preliminary data of the activity of HGPRT after initiating allopurinol in a small group of IBD patients were previously reported.
2.7. Hypoxanthine-guanine phosphoribosyl transferase (HGPRT)

The activity of HGPRT was measured in RBC and expressed in nmol of product per hour per mg protein (nmol/(h×mg protein). The RBC pellet was suspended in deionized water, sonicated and diluted in 250 mM Tris–HCl (pH 7.4) containing 25 mM MgCl₂. The HGPRT activity was measured in 20,000 g diluted supernatant, as described earlier.²⁶,²⁷ Final substrate concentrations were 1.86 mM 5-phosphoribosyl-1-pyrophosphate (PRPP) (Sigma-Aldrich Corp., St. Louis, MO, USA), with (18–28 μg protein per assay) and 0.15 mM [8-¹⁴C] hypoxanthine (47 mCi/mmol, Moravek Biochemicals Inc., Brea, CA, USA).

2.8. Thiopurine S-methyl transferase (TPMT)

The activity of TPMT was measured in RBC in pmols per hour per mg protein. Thiopurine S-methyl transferase activity in RBC was determined by a validated high-performance liquid chromatography (HPLC) as described earlier.²⁸

2.9. Xanthine oxidase (XO)

The activity of XO was measured in plasma by using the Amplex® Red Xanthine/Xanthine Oxidase Assay kit (Invitrogen, Carlsbad, CA, USA). Xanthine oxidase converts hypoxanthine in xanthine, and xanthine into uric acid, by both reactions, H₂O₂ is released. With H₂O₂ as cosubstrate, Horse radish peroxidase oxidizes Amplex Red to the detectable red-fluorescent oxidation product resorufin with absorption and fluorescence emission maxima of 571 and 585 nanometres, respectively. Plasma XO activity was expressed in mU per hour per ml, where 1 unit is defined as the amount of XO that will form 1 μmol of uric acid from xanthine.

3. Statistics

Continuous variables were expressed as median with interquartile range (IQR) and categorical variables were expressed as numbers and percentages. Correlations of quantitative variables were explored with the Spearman’s rho test. To assess differences in enzyme activities over time the Friedman test was used. Post-hoc analysis with Wilcoxon Signed-Rank Tests was conducted with a Bonferroni correction applied, resulting in a significance level set at p<0.017. For all other variables p<0.05 was considered statistically significant. SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

4. Results

4.1. Demographic characteristics

Fifteen patients, of whom twelve were females (80%), were included. Eleven patients (73%) were diagnosed with CD. The median age at initiating combination therapy was 40 years (IQR 29–57) and median duration of IBD was 1.5 year (IQR 0.8–12.0). Reasons to initiate combination therapy were hepatotoxicity, other adverse reactions, thiopurine resistance or a combination of hepatotoxicity and thiopurine resistance in 7 (47%), 4 (27%), 2 (13%) and 2 (13%) patients, respectively. The median duration of monotherapy with thiopurines was 5 months (IQR 2–33). During combination therapy thirteen patients were treated with 25 mg 6MP and two with 50 mg AZA daily. Four patients were concomitantly treated with 5-ASA, two of whom also used corticosteroids,
and one patient only used corticosteroids alongside combination therapy. The 5-ASA dose was stable during the study follow-up. All patient characteristics are depicted in Table 1.

4.2. Enzyme assays

Fig. 2 illustrates the different enzyme activities over time. The activity of HGPRT increased, from 150 (IQR 114–176) to 180 (IQR 135–213) after 4 weeks, and to 204 nmol/(h×mg protein) (IQR 173–213) after 12 weeks. This increase was statistically significant (p = 0.013 monotherapy compared to 12 weeks of combination therapy). TPMT activity was not altered by combination therapy and was 29.8 (24.1–35.8), 29.8 (28.0–30.8) and 31.8 pmol/hour/mg protein (30.3–35.2; Friedman p = 0.199) after 4 and 12 weeks, successively. The activity of XO decreased from 0.18 (IQR 0.08–0.3) during thiopurine monotherapy to 0.14 (IQR 0.06–0.2) after 4 weeks to 0.11 (IQR; 0.06–0.17 p = 0.008) mU/hour/ml after 12 weeks of combination therapy. There were no correlations regarding enzyme activities and thiopurine metabolites or toxicity variables.

4.3. Thiopurine metabolites

At 4 weeks of combination therapy median (IQR) 6-TGN concentrations were increased in all patients, from 138 (119–188) to 235 (223–304) and to 265 pmol/8×10^8 (188–344) after 12 weeks of combination therapy (p < 0.001). In contrast, both 6-MMPR concentrations and 6-MMPR/6-TGN ratios decreased in all patients (both p < 0.001). Median (IQR) concentrations of 6-MMPR decreased dramatically from 13230 (7130–17420) to 690 (378–1325), and 540 (240–790) pmol/8×10^8, respectively. Similarly, the median (IQR) 6-MMPR/6-TGN ratio decreased from 96 (54–120) to 1.1 (0.7–2.0) and 1.9 (0.6–2.9) at 4 and 12 weeks of combination therapy, successively.

4.4. Evaluation of haematological and liver toxicity

All haematological and liver test variables are depicted in Table 2. Leukocyte counts decreased from 7.5×10^9/L (IQR 4.8–7.9) during thiopurine monotherapy to 4.4 ×10^9/L (IQR 4.2–5.7) (p = 0.037) at 12 weeks of combination therapy. Concentrations of ASAT, ALAT, and AP decreased and yGT slightly increased during combination therapy, although not statistically significant (Table 2). Leukocytopenia (leukocyte count 0.9×10^9/L) occurred in one patient, who unintentionally continued full-dose AZA during combination therapy. The leukocyte count of this patient recovered spontaneously 2 weeks after discontinuing combination therapy of AZA and allopurinol. Subsequently, adequate combination therapy was initiated and well tolerated without any signs of recurrent myelotoxicity.

5. Tolerability of combination therapy

After a median duration of 4.5 weeks (IQR 1.8–8.0) three out of the initial fifteen patients (20%) failed combination therapy due to adverse reactions. Adverse reactions included nausea, fatigue and malaise. Two out of these three patients initiated combination therapy due to the same adverse reactions during thiopurine monotherapy. All adverse reactions were reversible upon cessation of combination therapy.

6. Discussion

In this prospective pharmacological drug interaction study, we observed possible effects of allopurinol-thiopurine
combination treatment on activities of key thiopurine metabolizing enzymes. In addition, we acknowledged the effect of allopurinol-thiopurine combination treatment on the formation of thiopurine metabolites in these IBD patients. A novel finding of this study is that upon allopurinol and low-dose thiopurine combination therapy HPRT activity increased. Xanthine oxidase activity decreased, but TPMT activity seemed not affected despite a steep decrease in the methylated thiopurine metabolite concentrations during combination therapy. In agreement with previous studies, a marked increase in 6-TGN concentrations was observed during combination therapy.12,29

The enhanced HPRT activity upon combination therapy may be explained by a direct inductor effect of allopurinol or one of its metabolites, since thiopurine therapy itself seems to decrease HPRT activity.30 The enhanced HPRT activity corroborates and extends preliminary data.25 Ding and colleagues recently reported a correlation between the activity of HPRT and 6-TGN concentrations, which was not observed in this study possibly due the small sample size.31 Nonetheless, the enhanced HPRT activity may at least in part explain the rise in 6-TGN concentrations as HPRT is the first enzyme, responsible for the generation of 6-TGN. Since HPRT is also involved in the formation of methylated 6-MP ribonucleotides, one would expect an increase in 6-MMPR.

However, in line with previous studies these metabolites decreased dramatically and disappeared almost completely. In analogy with a previous study no difference in HPRT activity was observed between gender and disease type (CD and UC).31

As a classical XO inhibitor, allopurinol decreased the activity of XO after initiating allopurinol. With regard to thiopurine metabolism, decreased XO activity, either by inhibition or by a decreased activity of XO, causes less shunting towards the XO pathway, thereby increasing the concentration of 6MP that remains available for conversion into 6-TGN but theoretically also for the conversion into 6-MMPR (Fig. 1).26,32 The latter is obviously not the case and the reason for this remains unsolved. The most logical explanation for the reduced 6-MMPR concentrations would be that allopurinol not only inhibits XO, but also TPMT, whether directly or indirectly by generation of an inhibiting metabolite.

Contrasting reports regarding TPMT inhibition by allopurinol have been published. Sparrow et al. quoted unpublished work by Prometheus Laboratories that showed that RBC TPMT activity was not inhibited by allopurinol in vitro.12 This finding has been disputed by others who argue that allopurinol is a prodrug that can only be converted to its active metabolite oxyxopurinol by XO and aldehyde oxidase (AOX), enzymes which are effectively absent in RBC. Oxyxopurinol is further converted to oxyxopurinol riboside monophosphate, which as a TPMT substrate may be responsible for (competitive) TPMT inhibition and the subsequent decrease in 6-MMPR production.33

In addition, there is evidence that 2-hydroxy-6-mercaptopurine (2OHMP) is a TPMT inhibitor.34 Also, 2OHMP can be formed from 6MP by AOX, especially when XO is inhibited (Fig. 1). Adding 2OHMP to a mixture of RBC and 6MP resulted in a significant reduction in RBC 6-MMPR production as compared to the mixture without 2OHMP.35

In line with two unpublished studies cited by Sparrow et al. our in vivo study did not show any alteration in TPMT activity upon allopurinol-thiopurine co-treatment.29 Yet, 6-MMPR concentration decreased substantially. However, the TPMT assay reflects the capacity of RBC to methylate thiopurines. Any inhibition by potential inhibitors may not be found, since they may not accumulate in RBC or if so, they may be diluted to below level of detection when preparing RBC for the TPMT assay.

Although we observed a marked effect of allopurinol in 6-TGN formation, it is not clear whether the combination is optimal and if a similar effect would be observed at lower doses of allopurinol. Study of other XO-inhibitors might therefore be of great interest.

Besides HPRT, inosine-5-monophosphate dehydrogenase (IMPDH) is also a rate limiting enzyme in the formation of the pharmacologically active 6-TGN. Previous studies, showed that patients with a high 6-MMPR/6-TGN ratio (above 20) had lower activity of IMPDH, compared to patients with a 6-MMPR/6-TGN below 20.38,39 Enhancement of IMPDH activity could thus increase 6-TGN concentrations. In addition, enhancement of the activity of guanosine monophosphate synthase (GPMS) may also provide an explanation for the
increased 6-TGN concentration (Fig. 1). As both enzymes contribute to 6-TGN production but not 6-MMPR, a decreased 6-MMPR/6-TGN ratio would be expected upon their induction. This needs further confirmation.

Another enzyme involved in thiopurine metabolism possibly related with a skewed thiopurine metabolism is inosine triphosphate pyrophosphohydrolase (ITPase). A single nucleotide polymorphism has been reported in association with a decreased ITPase activity. Genetic alterations in the ITPase gene, and thus a decreased ITPase activity, are associated with adverse outcomes of conventional thiopurine therapy, probably by the accumulation of 6-thioguanosine-triphosphate (6TITP). Since 6TITP is a substrate for TPMT, accumulation of 6TITP may result in accumulation of 6-MMPR (Fig. 1). Hence, ITPase deficiency might for a part be responsible for a skewed thiopurine metabolism. Whether ITPase induction contributes to high 6-TGN and low 6-MMPR concentrations observed during combination therapy remains to be established.

The ratio of 6-MMPR/6-TGN above 20 is arbitrarily defined but in line with other studies, however there is no widely accepted definition of a skewed thiopurine metabolism. The lowest 6-MMPR concentration in our study was 6220 pmol/8× 10^8, suggesting that all our included patients displayed an aberrant thiopurine metabolism.

Although all patients were strictly informed to decrease the thiopurine dose when initiating allopurinol, one patient still continued the full weight-based dose and had to discontinue therapy temporarily due to leukocytopenia (leukocyte count 0.9 ×10^9/L). This case illustrates the serious risks of therapy in patients with inflammatory bowel disease and normal TPMT activity.

In conclusion, HGPRT induction and XO inhibition seem related with a skewed thiopurine metabolism is inosine triphosphate pyrophosphohydrolase (ITPase). A single nucleotide polymorphism has been reported in association with a skewed thiopurine metabolism. Whether ITPase induction contributes to high 6-TGN and low 6-MMPR concentrations observed during combination therapy remains to be established.

Conflicts of interest statement

None.

References


