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Azathioprine Therapy in Multiple Sclerosis: Phosphoribosylated Metabolites and Thiopurine Methyltransferase Activity

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Abstract: *Objective:* In this prospective study, we examined the association between azathioprine dose, levels of its phosphoribosylated metabolites, and the activity of thiopurine methyltransferase in patients with multiple sclerosis (MS). *Materials/Methods:* Clinical data and blood samples were collected from 27 MS patients who were undergoing azathioprine treatment. In red blood cells, thiopurine methyltransferase (TPMT) activity was determined, and after hydrolysis and cleavage of the phosphoribosyl residue, amounts of 6-thioguanine (6-TG), 6-methyl-thioguanine (6-MTG), 6-methylmercaptopurine (6-MMP) were measured. For clinical evaluation, the expanded disability status score (EDSS) and the multiple sclerosis functional composite (MSFC) were performed. Laboratory and clinical examinations were conducted twice with a 6-month-intervall. *Results:* Over a broad range of daily azathioprine dose, nearly constant levels of the immunosuppressive-active 6-TG (nucleotides) were found. There was, however, a marked relationship between daily azathioprine dose and 6-MMP nucleotide levels. Especially patients receiving an azathioprine dose of more than 1.5 mg/kg per day in particular presented an exponential increase in 6-MMP levels when TPMT activity was higher than 45 U/g Hb. All the biochemical measurements gave similar results when performed 6 months later. *Conclusions:* Patients with the combination of a high TPMT-activity and an azathioprine dose of more than 1.5 mg/kg/d exhibit significantly increased 6-MMP nucleotide levels. These patients are thus at risk for hepatotoxic side effects. Determination of TPMT activity before azathioprine therapy and monitoring of its metabolites might provide guidance for dose individualization.

Keywords: Azathioprine, multiple sclerosis, phosphoribosylated metabolites, thiopurine methyltransferase activity.

INTRODUCTION

Azathioprine was introduced in MS therapy in the 1970s [1, 2]. Since then, several studies have shown its effectiveness in the treatment of MS [3-5]. Additionally, it is used in the immunosuppressive treatment of other neurological autoimmune diseases, for example, myasthenia gravis [6, 7]. Moreover, azathioprine is used widely in the treatment of other, non neurological, autoimmune and inflammatory conditions, for example rheumatoid arthritis (RA) or inflammatory bowel disease (IBD) [8, 9]. It is licensed for MS therapy in Germany, and today, azathioprine represents an established second-line therapy [10]. However, azathioprine predisposes to adverse events. Apart from idiosyncratic side effects such as fever or gastroenterological disturbances, there are severe dose dependent side effects, in particular hepatoxicity and myelodepression. Azathioprine is a prodrug and metabolized via a biochemical sequence, which is summarized in (Fig. **1**) [11]. The key-enzyme is thiopurine methyltransferse (EC: 2.1.1.67, TPMT) that exhibits an autosomal co-dominant polymorphism [12, 13]. 6-Thioguanine nucleotides (6-TG nucleotides) are thought to be the immunosuppressive-active metabolites [14]. 6-TG nucleotides are incorporated into DNA, results in inhibition of lymphocyte proliferation. Different studies on thiopurines in IBD showed that elevated levels of 6-TG nucleotides pose an increased risk of myelosuppression, whereas 6-methylmercaptopurine (6-MMP) nucleotides are related to an increased risk of hepatotoxicity [15]. As a result of the complex metabolism and genetic polymorphism of metabolic enzymes, there is wide interpatient variation in the concentrations of active and potentially toxic metabolites in patients under azathioprine therapy [16]. Despite these findings, traditional dosing strategies for azathioprine in MS therapy are based on weight or are empirically chosen. These strategies do not take into account the considerable interindividual differences in azathioprine metabolism. The objective of this study was to investigate levels of phosphoribosylated azathioprine metabolites as a function of daily dose and TPMT activity in MS patients.

MATERIALS AND METHODS

Patients and Clinical Assessment

This prospective study included 27 patients with definite MS (5 men, 22 women, mean age 49.1 years, range 27-70). Subjects were recruited from the Department of Neurology at the University of Luebeck, Germany. Further inclusion criteria were age of eighteen years or older and treatment with a constant daily dose of azathioprine for at least six months prior to the study. The study procedure included examination by trained neurologists using the extended disability status scale (EDSS) and the multiple sclerosis functional composite (MSFC) [17, 18]. Blood samples were drawn at

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two points of time (T1 and T2) with an interval of about six-months. Other clinical data collected included course of MS, sex, age at time of diagnosis, dose of azathioprine, and body weight. The study was conducted according to the ethical standards laid down in the 1964 Declaration of Helsinki and was approved by the local ethics committee. Before partici-pation, informed written consent was obtained from all par-ticipants.
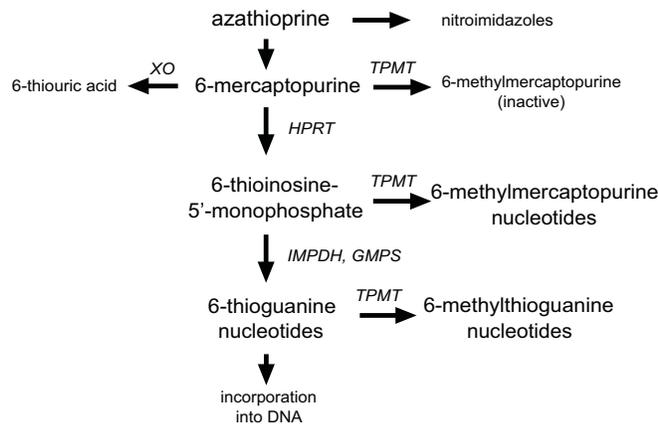
Fig. (1). Azathioprine metabolic pathway. Simplified azathioprine metabolism according to Karran & Attard [11]. TPMT: Thiopurine methyltransferase. XO: Xanthine oxidase. HPRT: Hypoxanthine guanine phosphoribosyl transferase. IMPDH: Inosine monophos-phate dehydrogenase. GMPS: Guanosine monophosphate synthase

TPMT

TPMT activity was determined using 6-thioguanine as substrate. We measured the formation of 6-methylthioguanine with S-adenosyl-methionin as the methyl donor. Quantitative measurement of 6-methylthioguanine was performed with HPLC and fluorescence detection. One unit of enzyme activity represents the formation of 1 nmol of 6-methylthioguanine per hour [19].

Azathioprine Metabolites

Amounts of 6-methylmercaptopurine (6-MMP), 6-thioguanine (6-TG), and 6-methylthioguanine (6-MTG) were measured after hydrolysis and cleavage from the phosphori-bosylated residue in red blood cells (RBC) by HPLC tech-niques [20]. Assay precision was measured using internal quality controls to ensure validity and reliability.

Statistics

Statistical analysis was performed using SPSS. Descrip-tive analysis included frequency, mean, standard error of mean, standard deviation of mean, median, minimum and maximum. Medians were given for better comparability with the clinical scores. Different statistical methods (ANOVA, and post hoc t-test for unpaired samples) were used when appropriate, logistic regression was used to adjust for poten-tial cofounders. P was significant at 0.05.

RESULTS

Study Group Patients

A total of 27 MS patients were recruited for the study. All patients were on azathioprine treatment for at least 6

months before participating in the study. Patient details are summarized in (Table **1**). There was no overall significant change of EDSS or MSFC over the period of time between the first and second visit (Table **2**). Also, TPMT activity and the concentrations of the metabolites did not change between T1 and T2 (Table **2**).

Table 1.   Characteristics of the 27 multiple sclerosis patients treated with azathioprine at 1st visit. *Values are median (range).

Sex (F:M)	22:5
Relapsing Remitting	16
Relapsing Remitting secondary progressive	9
Primary Progressive	2
Age (years) *	49 (27 - 70)
Duration of MS (years) *	14 (6 – 40)
Age at onset of MS (years) *	34 (21 – 49)
weight (kg) *	66 (47 - 107)
BMI (kg/m^2) *	24.1 (16.7 – 32)
Azathioprine dose (mg/day) *	100 (50 – 200)
Azathioprine dose (mg/kg/day)*	1.7 (0.5 – 2.7)

TPMT

TPMT activity was measured twice, with an approximate time interval of six months. Values showed a Gaussian dis-tribution. Mean enzyme activity was slightly higher after six months (T1 mean 34.4 ± 1.7; T2 mean 41.9 ± 1.34), however without significant difference (p=0.202; Table **2**). All pa-tients were in the homozygous high range (>14 U/g Hb). TPMT activity did not correlate with patients' age or disease duration (data not shown). Enzyme induction by azathioprine could not be observed since TPMT activity was not related to azathioprine dose. ANOVA gave R^2 value of 0.077 which means that the azathioprine dose accounts for only 7.7 % of the activity variance.

Azathioprine Metabolites and Dose

Amounts of phosphoribosylated azathioprine metabolites (nucleotides) were quantified after acid hydrolysis. **6-Thioguanine** (6-TG) was measured twice in red blood cells (ng/g Hb) (Table **2**). Initial median [range] erythrocyte 6-TG levels (415, [192 -1174]) did not significantly change during the months of treatment (461, [132 – 1749]). 6-TG showed only a slight tendency, but not significant enhancement when daily azathioprine dose increased for patients, with spearman coefficient of r=0.087 (p=0.678) (Fig. **2a**).

Levels of **6-methylthioguanine (**6-MTG) clearly in-creased with daily azathioprine dose at T1. The relationship appeared linear and was statistically significant with spear-man coefficient of r=0.47 (p=0.018). ANOVA also showed significant variance of regression (p=0.006) (Fig. **2b**).

Table 2.   Clinical scores, TPMT activity and azathioprine metabolites of patients at T1 and T2.

Parameter	T1 (1st Visit)	T2 (2nd Visit)	P value
EDSS (points) median (range)	2.5 (1 – 7)	2.5 (0-7)	0.737
MSFC (Z-Score) median (range)	-0.07 (-0.92 – 4.64)	-0.04 (-0.71 – 0.71)	0.559
TPMT activity median (range), U/g Hb	43 (21 – 57)	42 (23-54)	0.202
6-TG median (range), ng/g Hb	415 (192-1174)	461 (132 – 1749)	0.178
6-MTG median (range), ng/g Hb	411 (45 – 896)	389 (62 – 3165)	0.615
6-MMP median (range), ng/g Hb	9407 (904 – 59149)	7423 (408 – 37098)	0.101

Taken from all patients, marked enhancement of **6-methylmercaptopurine** (6-MMP) levels was observed when high doses of azathioprine were given. In contrast to 6-MTG, the relationship appeared as an exponential growth (ANOVA p=0.001; Fig. **2c**).

Effect of Azathioprine Dose and TPMT Activity on Nucleotide Levels

To evaluate combined effects of azathioprine dose and TMPT activity on nucleotide levels, patients were divided into four subgroups: patients with low TPMT activity and low azathioprine dose (group A, n=6), patients with high TPMT activity and low dose (group B, n=3), patients with low TMPT activity and high dose (group C; n=12) and patients with high TMPT activity and high dose (group D, n=6). Since the median of TPMT activity of all patients was 43 U/g Hb, we set the limiting value for TPMT activity as 45 U/g Hb. Limiting value for the dose was 1.5 mg/kg per day. As shown in (Table **3**), there were no statistically significant differences in 6-TG or 6-MTG nucleotide levels between groups. Lowest 6-MMP nucleotide levels were found in group A. In group B, amounts of phosphoribosylated 6-MMP were moderately enhanced. In group C patients (low TPMT activity but high azathioprine dose of more than 1.5 mg/kg per day), 6-MMP levels were three times higher compared to group A. However, highest 6-MMP levels could be observed in group D (patients with high TMPT activity and high dose). Here, levels were approximately 12 times higher than in group A patients. Fig. (**2d**) illustrates these findings with different exponential growth curves according to high or low TMPT activity.

DISCUSSION

To our knowledge, this is the first study that presents azathioprine data to metabolites and thiopurine methyltransferase (TPMT) activity in MS patients who are on long term treatment. Our results showed marked relationship between daily azathioprine dose and levels of 6-methylmercaptopurine (6-MMP) nucleotides in MS patients predominantly with TPMT-activity higher than 45 U/g Hb. Most studies concerning the use of 6-mercaptopurine (6-MP) of which azathioprine is the prodrug, and measurement of the metabolites that have been conducted in patients with inflammatory bowel disease (IBD) [21, 22]. It has been noted that some patients with IBD did not respond to an immunosuppressive therapy with azathioprine. Dubinsky *et al.* could demonstrate that these patients did not exhibit significant increase of the immunosuppressive active metabolite 6-thioguanine (6-TG) nucleotide despite escalation of azathioprine doses. Since, in contrast to 6-TG nucleotides, amounts of phosphoribosylated 6-MMP significantly increased, they concluded that some IBD patients preferentially metabolize 6-MP to 6-MMP rather than to 6-TG nucleotides [23]. Our data may confirm these findings also for MS patients. According to our data, this subgroup consisted of patients with normal to high TPMT activity and also high azathioprine dose treatment. We suppose that in those patients high enzyme activity "shunts" the azathioprine metabolism to 6-MMP nucleotides. Further increase of a daily azathioprine dose would lead to an enhancement of 6-MMP nucleotides, however, without significant increase of 6-TG nucleotides. Here, TPMT may stabilize levels of 6-TG nucleotides over a broad range of azathioprine doses by methylation to phosphoribosylated 6-MMP and 6-MTG (see Fig. **1**). Therefore, two important points can be derived from this fact for clinicians. If patients with high TPMT activity (probably higher than 45 U/g Hb) fail to respond to azathioprine treatment, especially at high doses (e.g. 1.5 mg /kg/d), further escalation would probably not lead to improvement. In this case, however, the risk of hepatotoxic side effects increases since the phosphoribosylated metabolite 6-MMP is clearly associated with hepatotoxicity [24]. Considering that on one hand 6-TG nucleotides are thought to be the predominant immunosuppressive active metabolites of thiopurines and on the other hand elevated 6-MMP nucleotides may result in hepatotoxic effects, we propose a maximum daily azathioprine dose of 1.5 mg/kg. In this context, Asseldonk *et al.* described IBD patients who had experienced hepatotoxicity due to 6-MMP nucleotides by treatment with azathioprine or 6-mercaptopurine. After switching the therapy to 6-thioguanine, immunosuppressive substances enter the metabolic pathway "further down", and a "shunting away" to 6-MMP nucleotides by TMPT is avoided. This strategy resulted in a reduced hepatotoxicity rate [25]. On the other hand, prior to any azathioprine treatment enzyme activity of TPMT should also be detected since patients with reduced enzyme activity exist, e.g. with other than homozygotic TMPT gene background [26]. Patients with a low TMPT activity were prone to develop myelotoxic side effects due to immunosuppressive active 6-TG nucleotides, while amounts of 6-MMP nucleotides were rather low [24, 27]. Since in our study enzyme activities ranged from normal to high values, these data can not support findings related to 6-TG
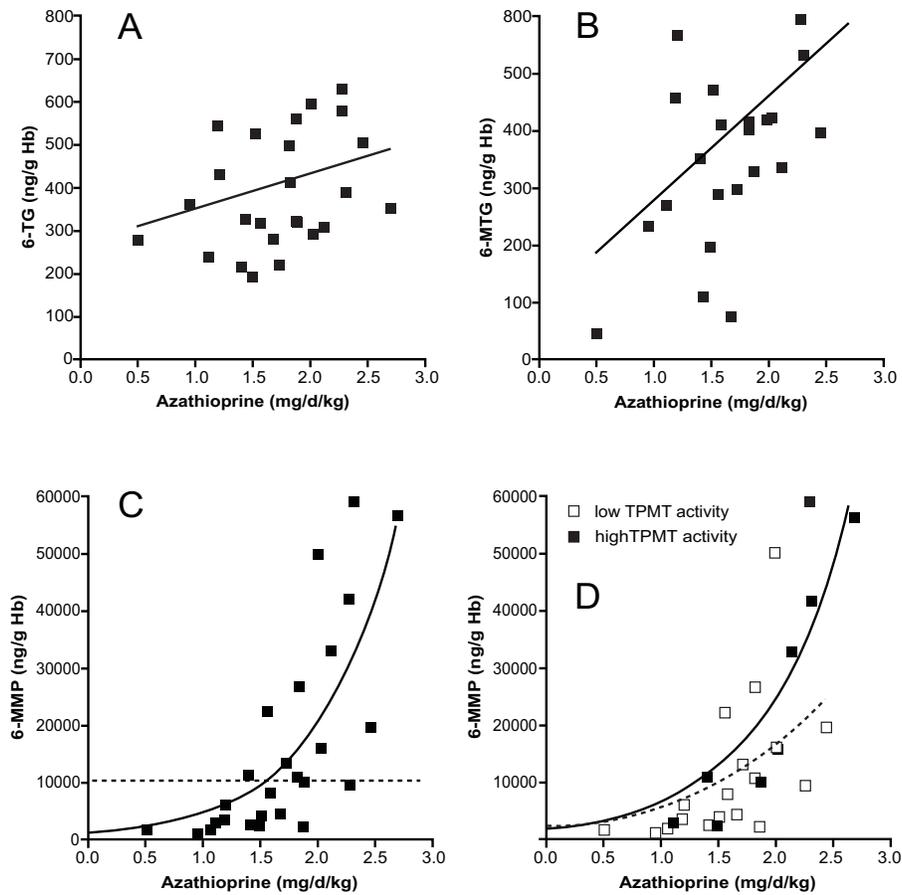
Fig. (2). Relationship between daily azathioprine dose and nucleotide levels of 6-thioguanine (6-TG; **A**), 6-methylthioguanine (6-MTG; **B**), and 6-methylmercaptopurine (6-MMP; **C** and **D**). Compounds were detected by HPLC technique after hydrolysis of the phosphoribosylated nucleotides. High TPMT activity was defined as enzyme activity of more than 45 U/g Hb (D).

Table 3.   **Azathioprine metabolite levels in patients with low TPMT and low dose (group A, n=6), patients with high TPMT activity and low dose (Group B, n=3), patients with low TMPT activity and high dose (Group C; n=12) and patients with high TMPT activity and high dose (Group D, n=6). Limiting value for daily azathioprine dose: 1.5 mg/kg, limiting value for TPMT activity: 45 U/g Hb. *p<0.05 significant, when compared to Group A. **p<0.05 significant, when compared to Group C.**

	6-TG (ng/gHb)	6-MTG (ng/gHb)	6-MMP (ng/gHb)
Group A (low TPMT, low dose; n=6) mean ± SD (min.-max.)	518 ± 136 (279 – 1174)	346 ± 103 (45 – 664)	2835 ± 747 (1049 – 6086)
Group B (high TPMT, low dose; n=3) mean ± SD (min.-max.)	215 ± 13 (192 – 240)	273 ± 44 (197 – 352)	5482 ± 2745 (2494 – 10966)
Group C (low TPMT, high dose; n=12) mean ± SD (min.-max.)	480 ± 53 (221 – 852)	405 ± 42 (75 – 669)	15491 ± 3875 (904 – 50236)*
Group D (high TPMT, high dose; n=6) mean ± SD (min.-max.)	427 ± 55 (308 – 630)	569 ± 90 (330 – 896)	33886 ± 9622 (2297 – 59149)**

nucleotides in the case of low TPMT activity, e.g. lower than 14 U/g Hb.

Our study has, however, additional limitations. The MS patient group is rather small and heterogenous. These factors cause statistical restrictions especially regarding the interpretation of the clinical data. Subsequently, the information our study can give, is the fact that the group represents a typical sample of MS patients. It is not possible to link clinical data in any way to azathioprine dose, TPMT activity or the metabolites levels. In particular it is not possible to draw any conclusions concerning the impact of these factors on the clinical response (or the lack of it). The patients' azathio-

prine doses were indeed not standardized. Yet, the data described above led us to suggest that after a 6 month stabilization period of individual treatment, TPMT activity as well as azathioprine metabolite levels are rather stable over time. Most findings regarding azathioprine therapy and analysis of metabolites were gained in studies on IBD or other internal diseases but not on Multiple Sclerosis. It is unclear, to which extent this knowledge can be transferred to the treatment of MS patients.

CONCLUSION

We could observe a strong relationship between daily azathioprine dose and hepatotoxic side-effect-inducing 6-MMP nucleotides in MS patients with high TPMT activity. Thus, we would propose measurement of TPMT activity before and phosphoribosylated metabolites measurements during azathioprine treatment in order to reduce toxic side effects. Further studies are needed to optimize azathioprine therapy and to evaluate the clinical impact of this compound on Multiple Sclerosis. Additionally, with teriflunomide inhibiting dihydroorotate dehydrogenase (EC 1.3.3.1) and pyrimidine biosynthesis, the therapeutical approach of DNA subunit modification is now renewed in treatment of Multiple Sclerosis.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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PATIENT'S CONSENT

Declared none.

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