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Abstract

Thiopurine methyltransferase (TPMT) is an important enzyme in the metabolism of 6-mercaptopurine (6MP), which is used in the treatment of acute lymphoblastic leukemia (ALL). TPMT catalyzes the formation of methylthioinosine monophosphate (MetIMP), which is cytotoxic for cultured cell lines, and it plays a role in detoxification of 6MP. Population studies show a genetic polymorphism for TPMT with both high and low activity alleles. About 1 of 300 subjects is homozygous for the low activity. The function TPMT plays in detoxification or therapeutic efficacy of 6MP in vivo is not clear. In this article the genetic polymorphism of TPMT is reviewed and the contribution of TPMT to the cytotoxic action, or detoxification, of 6MP in children with ALL is discussed. Induction of TPMT activity has been described during the treatment for ALL. We performed a pilot study on the influence of high-dose 6MP infusions (1300 mg/m² in 24 h) on TPMT activity of peripheral blood mononuclear cells (pMNC) of eleven patients with ALL. The TPMT activities were in, or, above the normal range. There was no statistically significant difference between the TPMT activities before and after the 6MP infusions. MetIMP levels in pMNC increased during successive courses. This might be explained by TPMT induction, but other explanations are plausible as well. Twenty five percent of the TPMT assays failed, because less than the necessary 5-10⁶ pMNC could be isolated from the blood of leukopenic patients. Red blood cells cannot be used for TPMT measurements, since transfusions are frequently required during the treatment with 6MP infusions. Therefore, the influence of high-dose 6MP infusions on TPMT activity can only be investigated further when a TPMT assay which requires less pMNC has been developed.

Keywords: Enzymes; Thiopurine methyltransferase; 6-Mercaptopurine

1. Involvement of thiopurine methyltransferase in the metabolism of 6-mercaptopurine

Thiopurine methyltransferase (TPMT) is involved in the methylation reactions of 6-mercaptopurine (6MP). Since 1953, 6MP has been administered in the treatment of childhood acute lymphoblastic leukemia (ALL) and is converted by several metabolic steps (Fig. 1). 6MP is catabolized by xanthine oxidase into inactive thioxanthine and thiouric acid which are excreted by the kidneys [1-4]. In the anabolic pathway, 6MP is first converted into thioinosine monophosphate by hypoxanthine guanine phosphoribosyltransferase (HGPRT). To have a cytotoxic effect this compound is either converted into a thioguanine nucleotide or it is methylated by TPMT into methylthioinosine monophosphate (MetIMP). Incorporation of thioguanine nucleotides (TGN) into DNA and RNA and inhibition of the de novo synthesis of the purine by MetIMP both result
methylmercaptopurine riboside (MeMPR) which can be phosphorylated intracellular MetIMP by adenosine kinase [12,13] and not be broken down by purine nucleoside phosphorylase [14]. The methylation by TPMT of 6-methylmercaptopurine (MeMP), which cytotoxic action, results in detoxification of [3].

In conclusion, TPMT is an important enzyme in the metabolic pathways of 6MP. It catalyzes the formation of MetIMP, which is an inhibitor of de novo synthesis of the purine in vitro, and plays a role in detoxification of the drug.

2. Genetic polymorphism of TPMT

In view of its role in thiopurine metabolism, it became important to study the genetic background of TPMT activity. These studies were facilitated by the development of a quantitative assay for TPMT activity in red blood cell (RBC) lysates [1]. The assay measures the transfer of UC from S-adenosyl-L-methionine to 6MP.

It became apparent that a marked variation in TPMT activity exists between individual subjects. The frequency distribution of TPMT activity in a population of white subjects is tri-modal. The subjects displayed high activity, intermediate activity, while the lysates of a small number of subjects contained no detectable TPMT activity.

The model for such a distribution is that a single autosomal locus governs TPMT activity and that the alleles for the locus [17]. One allele corresponds to the high activity form (TPMT H), and the other to the low activity form (TPMT L). Homozygotes have high activity, heterozygotes have intermediate activity, and homozygotes have low activity.

The model was suggested by the distribution of TPMT activity with Hardy-Weinberg statistics for the TPMT locus. The frequency of TPMT H in the group of white subjects studied. The model was strongly supported by agreement with Mendelian laws. The frequency of TPMT L (0.06 for TPMT H) in the group of white subjects studied. The model was strongly supported by agreement with Mendelian laws. The frequency of TPMT L (0.06 for TPMT H) in the group of white subjects.
in agreement with Mendelian rules. Further studies and sophisticated statistical analyses led to the conclusion that TPMT activity is governed by a single locus, although additional circumstances may influence TPMT activity [18,19].

Individual levels of RBC-TPMT activity correlate with the levels of TPMT activity found in other tissues, including liver, kidney, lymphocytes, platelets and lymphoblasts [20–23]. Apparently, the same genetic locus controls TPMT activity in these tissues. TPMT activity has further been detected in all other tissues examined: i.e., brain, lung, intestine and placenta [24]. The liver and kidney had the highest levels of TPMT activity [24].

Age does not have an effect on TPMT activity and high levels of TPMT activity have been found in fetal tissues [24]. It has been reported that tissues derived from males have approximately 10% higher levels of TPMT activity than those from females [19,23,25], but other studies do not find significant sex differences [26–28]. Uneven frequency distributions of TPMT activity are also found in diverse ethnic groups [26,28]. The frequency of TPMTL was 0.02 in a group of Chinese subjects [28]. Differences in the activity levels between ethnic groups may be caused by laboratory circumstances [28]. However, when measured at the same laboratory, the levels of TPMT activity in samples from black subjects were 17% lower than those from white subjects, while similar proportions of high and intermediate activity were present in both groups [29].

Molecular biological studies were initiated with the partial purification of TPMT and production of specific antibodies [22]. TPMT is a cytoplasmic protein of 35 kDa [30,31]. Titration of the TPMT activity with antibodies indicated that variation of the TPMT activity between individuals corresponds to variation in the amount of TPMT protein [22].

The TPMT activity from kidney homogenates was resolved in two peaks by ion-exchange chromatography on DEAE-Sepharose [31]. These two activities behaved identically in all further experiments, which included kinetic studies, size exclusion chromatography, gel electrophoresis and column-chromatography on hydroxylapatite [31,32]. The nature of the difference in these isoenzymes is not known. Studies using individual samples clearly ruled out any link between genetic polymorphism and the existence of the two isoenzymes [31].

Extensive purification of TPMT was achieved after UV cross-linking of the protein to its labeled co-factor [3H-methyl]S-adenosyl-L-methionine. TPMT could thus be monitored by measuring its radioactivity instead of its enzyme activity [32].

A cDNA of human TPMT mRNA was cloned using peptide sequences of purified protein fragments [33,34]. A liver cDNA of 1.8 kb was cloned that has an open reading frame of 245 amino acids [34]. The identity of this cDNA was confirmed by expression of the coding region in COS cells, which resulted in an increase of TPMT activity [33]. The derived amino acid sequence of TPMT lacks the three regions that are commonly found in other S-adenosyl-L-methionine binding proteins [33]. It will be interesting to see whether the methyl donor is a co-factor for the physiological function of this protein.

The gene structure, including its regulatory sequences has not been resolved yet. Cloning of the gene is obstructed by the presence of a processed pseudo-gene on chromosome 18q21 [34]. The pseudo-gene originates from a reverse transcription event with TPMT mRNA.

The cDNA of one patient with TPMT-deficiency was analyzed [35]. Cloned cDNA from this patient was mutated in the open reading frame, leading to an amino acid substitution of Ala at position 80 to Pro. The corresponding cDNA was expressed in yeast and, contrary to the wildtype cDNA, the mutant protein had no TPMT activity. The pedigree of this patient indicates that the point-mutation accounts for only one copy of the gene, as the second copy seems to be affected by another mutation.

Mutations that affect TPMT activity may leave the physiological activity unchanged. Since TPMT is present in all tissues examined, from an early stage of development, it may represent an important or essential household protein. Knock-out and mutation experiments in mice should be performed to test these ideas.

The preliminary DNA sequence data indicate that different mutations are responsible for the TPMTL allele. Clearly, an inventory of existing mutations that lead to TPMT-deficiency is required.
3. TPMT and its contribution to the cytotoxic action or detoxification of 6MP

The genetic polymorphism of TPMT is not apparent in healthy subjects, but becomes important when thiopurine drugs, which are metabolized by this enzyme, are administered. Several patients developed signs of severe toxicity following 6MP treatment and it was demonstrated that they were deficient in TPMT activity [36–38]. Apart from its influence on 6MP toxicity, TPMT activity also appears to play a role in the prognosis and survival of childhood ALL, reflecting its importance in the therapeutic efficacy of 6MP. All studies that reveal insight into the efficacy of 6MP, and into the relevance of each cytotoxic pathway of 6MP in vivo, are performed in RBC during 1.5 to 2.5 years of maintenance therapy (MT) for childhood ALL, where low daily oral doses of 6MP (50–100 mg/m^2) and weekly doses of methotrexate (MTX) are administered [36–44]. Lymphoblasts, whose levels of TPMT activity correlate with the levels of activity found in RBC [20] are not available for investigation, because the patients are in remission during MT. Several studies performed with 6MP during MT suggested that the formation of TGN is mainly responsible for the treatment-outcome and for the drug toxicity [36–42]. This is supported by a significant positive correlation between high TGN levels in RBC and thiopurine induced bone marrow toxicity [41] and by negative correlations between TGN concentrations and TPMT activity [39] or between TGN concentrations and the incidence of relapse [39,40,42]. MetIMP levels were not measured in the latter studies. Another study with RBC showed that MetIMP and TGN concentrations, as well as levels of TPMT activity, were below the population median in six out of the seven patients who relapsed [43]. This raises the question whether low levels of TGN alone, or in combination with low MetIMP levels, in RBC during MT are responsible for the worse prognosis.

Evidence exists that the methylation of 6MP, catalyzed by TPMT, modulates 6MP cytotoxicity and that the production of TGN alone is not sufficient to explain the cytotoxic action of 6MP in vivo. TPMT-deficient patients, in whom methylated metabolites could not be detected in RBC, did not suffer from toxicity after the dose of 6MP was reduced considerably, but still had elevated TGN levels compared to patients with unimpaired 1 activity [36,38]. The tolerance of higher TGN concentrations, without bone marrow depression, TPMT-deficient patients suggests that the methylied metabolites of 6MP also contribute to cytotox: This is also illustrated by the preliminary result recent study that showed identical effects on marrow toxicity, in patients who were treated either 6MP or 6-thioguanine. However, the concentrations found in patients treated with were five times lower than those found in patients treated with 6-thioguanine [44]. So, in addition formation of TGN, the methylated metabolites of 6MP intracellularly may also contribute to marrow toxicity in vivo.

It is important to further elucidate whether methylation of thiopurines contributes to the cytotoxic action of 6MP in vivo, because this may clinical implications for the administration of 6-thioguanine, which is converted directly into 6MP. If methylation of 6MP only results in detoxification of the drug, it would be better to administer thioguanine instead of 6MP, especially in patients with high TPMT activity. If the formic MetIMP adds to the cytotoxic action of 6MP, TPMT activity would enhance the therapeutic activity of 6MP in the lymphoblasts. The cytotoxicity could be inhibition of the de novo synthesis of the would be lost if 6-thioguanine was administered instead of 6MP, because MetGMP is a twelve less potent inhibitor of the de novo synthesis of purine than MetIMP [45].

4. Induction of TPMT activity

The levels of TPMT activity in RBC during childhood ALL were higher compared to the diagnosis [20] and those of healthy controls TPMT activities showed low intra-patient vari (13.5%) during MT and were not influence high-dose MTX infusions [20]. After cessation MT the elevated levels of TPMT activity in decreased to normal levels [20,39]. The first of another study showed that the levels of activity in RBC were elevated at the end intensive induction and consolidation treatmener
just before the start of MT, and that they decreased during MT compared to the levels observed just before the start of the MT [46]. These observations suggest that induction of the TPMT activity in RBC occurs during the induction, consolidation or MT of childhood ALL [20,39,46]. It is not known whether the induction of TPMT activity also occurs in other tissues or in lymphoblasts, but the latter would be difficult to investigate because no lymphoblasts are available as soon as the patient is in remission. The biochemical mechanism for the induction of TPMT activity is not known.

High-dose 6MP infusions are currently being investigated in the induction and consolidation treatment of childhood ALL [47]. Since methylation of 6MP and its metabolites occurs to a large extent, especially during high-dose 6MP infusions [36,48,49] and, since 6MP infusions are administered over several courses [47], it is important to investigate the influence of high-dose 6MP infusions on the TPMT activity. In order to evaluate the consequences of TPMT induction on the therapeutic effect of 6MP, it is essential to elucidate the contribution of the methylation pathway to 6MP cytotoxicity, or detoxification, in vivo.

4.1. Effects of high-dose 6MP infusions on the activity of TPMT (pilot study)

A pilot study was performed to investigate the effect of high-dose 6MP infusions on the activity of TPMT and to explore the relationship between this activity and (methyl)thiopurine nucleotide levels. In the consolidation treatment using high-dose MTX infusions, of the Dutch Childhood Leukemia Study Group (ALL 8 study), patients with ALL were randomly divided into two groups; the first group were given high-dose 6MP intravenously and the second group received low-dose 6MP. The group that were given low-dose 6MP orally were treated as the control group for the high-dose 6MP group, because both groups received MTX infusions which may influence TPMT levels as well. We analyzed peripheral blood mononuclear cells (pMNC), instead of RBC, because the patients frequently received RBC transfusions for anemia. This pilot study was the first study in ALL which investigated both the activity of TPMT and the metabolism of 6MP in pMNC during treatment with high-dose 6MP infusions.

4.2. Experimental

Twenty children (aged between 2 and 9 years) with medium-risk ALL were treated in our center. After induction of bone marrow remission, all patients received the M-protocol, a consolidation treatment which consisted of four courses of treatment with high-dose MTX infusions (5 g/m² over 24 h) over 8 weeks. In addition, eleven of these patients were randomly selected for intravenous administration of 6MP (1300 mg/m² over 24 h given immediately after the MTX infusion i.e., from 24 to 48 h). The other nine patients received 6MP orally (25 mg/m² daily over 8 weeks). Patients or parental approval were obtained according to the guidelines of the ethical committee of our hospital.

pMNC were isolated from 20 ml of defibrinated blood by density-gradient centrifugation and contaminating RBC were removed by a NH₄Cl shock [50]. pMNC were isolated for measurement of TPMT activity before, and 2 weeks after, the M protocol. TPMT activity was measured according to the method of Van Loon and Weinshilboum [21], which is based on the methylation of 6MP with S-adenosyl-1-[¹⁴C]-methionine. In short, 5·10⁶ pMNC were incubated with a solution consisting of potassium phosphate buffer, pH 7, 6MP in dimethylsulfoxide, S-adenosyl-1-[¹⁴C]-methionine, reduced glutathione and allopurinol. The incubation was performed in a shaking water bath at 37°C for 90 min after which time the reaction was terminated using borate buffer, pH 10.1-[¹⁴C]-MeMP was extracted from the reaction mixture using 20% isoamyl alcohol in toluene. Radioactivity was measured for 10 min in a liquid scintillation counter and corrected for 3 separate blanks as described [21].

During the four courses of treatment, pMNC were isolated before and 24, 28, 48, 52 and 72 h after the start of each MTX infusion from eight patients treated intravenously and four patients treated orally. The (methyl)thiopurines were extracted, from pMNC (on ice), using perchloric acid and the pH was adjusted to between 6 and 7 with dipotassium hydrogen phosphate. The (methyl)thiopurine nucleotides were separated by anion-exchange HPLC.
(Partisil-10-SAX, 250 × 4.6 mm I.D., particle size 10 μm, Whatman, Clifton, NJ, USA) and measured the UV absorbance was measured at 290 nm for MetIMP and at 320 nm for thiopurine nucleotides as described [50].

4.3. Results and discussion

The activity of TPMT in pMNC was measured before and after the M protocol (Table 1). Before the start of the M protocol the levels of activity of TPMT (range 4.6–27.2 IU/10⁹ pMNC, mean 13.7) were in, or above, the range found in the lymphocytes of healthy adults (range 4.8–17.7 IU/10⁹, mean 11.0) [21]. The higher levels of TPMT activity found might be caused by other chemotherapeutic drugs used during the induction treatment [20,39,46].

There was neither a statistically significant difference between the levels of TPMT activity found before, or after, the M protocol in either groups (paired t test p = 0.50 in the intravenous group and p = 0.94 in the oral group) nor in the TPMT activity obtained after the M protocol between the groups (Mann–Whitney–Wilcoxon rank sum test p = 0.77).

The necessary 5·10⁶ pMNC for the TPMT could not be obtained in 9 out of 36 cases, by several patients were leukopenic. For future re-in ALL, an assay for the TPMT activity, requires less pMNC will be needed. We re-reported a non-radiochemical HPLC procedure for other purine enzymes using 1500–15000 pMNC [51], but we still have not been able to develop a sensitive HPLC assay for measuring TPMT activity that requires less than 5·10⁶ pMNC. Immunological techniques to detect the TPMT protein may be a sensitive alternative to the measurement of activity [22]. However, more insight into the genetic basis for TPMT deficiency in the population is required before this technique can be used reliably.

In the group treated intravenously, MetIMP (Table 2) were measurable at the end of the infusion (48 h) and either increased or remained constant after termination of the infusion from 48 h. Patient 3 did not produce any MetIMP during courses of treatment although his TPMT activity was 16.7 IU/10⁹ pMNC after the M protocol. The patients produced significantly more MetIMP during Course 4, compared to Course 1 (paired t test p = 0.004, eleven patients). During the subsequent courses of treatment, patients 5 and 7 produced MetIMP earlier (i.e., at 48 h instead of 52 or 72 h). During Course 4, patient 5 produced MetIMP earlier as 10 pmol/10⁶ pMNC (28 h). In the group treated by oral administration, MetIMP levels could be detected. Thiopurine nucleotides were not detected in both groups [detection limits are 1 and 3 pmol per injection (50)].

In the group treated intravenously, there was a correlation between the levels of TPMT activity before the start of the M protocol and the MetIMP levels after 72 h of the first course of treatment. Between the levels of TPMT activity after protocol and MetIMP levels during the fourth
Table 2
MetIMP levels in pMNC (pmol/10^6) over four courses of treatment from patients treated with HD-MTX followed by HD-6MP infusions.

<table>
<thead>
<tr>
<th>Patient</th>
<th>TPMT</th>
<th>MetIMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td></td>
<td>(72h)</td>
<td>(72h)</td>
</tr>
<tr>
<td>1</td>
<td>12.9</td>
<td>n.d.*</td>
</tr>
<tr>
<td>2</td>
<td>14.4</td>
<td>7.6</td>
</tr>
<tr>
<td>3</td>
<td>n.d.</td>
<td>10.6</td>
</tr>
<tr>
<td>4</td>
<td>17.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>5</td>
<td>22.5</td>
<td>29.4</td>
</tr>
<tr>
<td>6</td>
<td>27.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>7</td>
<td>9.7</td>
<td>3.5</td>
</tr>
<tr>
<td>8</td>
<td>7.8</td>
<td>4.1</td>
</tr>
<tr>
<td>Mean</td>
<td>8.5</td>
<td>13.7</td>
</tr>
<tr>
<td></td>
<td>(3.1)</td>
<td>(3.9)</td>
</tr>
<tr>
<td>Paired t test (p) compared to Course 1</td>
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</tr>
</tbody>
</table>

Levels found 24 h after termination of the 6MP infusion (72 h) are indicated. TPMT activities (IU/10^6 pMNC) before and after the M protocol are also indicated.

"n.d. = not done.

of treatment (Spearman's correlation coefficients 0.029 and -0.400, respectively). However, a larger number of patients would have to take part in the study before a conclusion could be drawn.

The significant increase in the levels of MetIMP in each patient during the successive courses of treatment with high-dose 6MP and the acceleration of MetIMP formation during the infusions over successive courses of treatment, in patients 5 and 7, suggest that there might be induction of TPMT activity by high-dose 6MP. This observation needs to be confirmed by measuring the levels of TPMT activity in more patients, because higher MetIMP levels could also be caused by other enzymes, which are involved in the metabolism of 6MP (Fig. 1). HGPRT activity in RBC was significantly higher in children during MT for leukemia compared to healthy control children [52]. An induction of HGPRT during chemotherapy might be another explanation for the increasing MetIMP levels in our study (Table 2), but HGPRT activity has never been investigated during high-dose 6MP infusions. Xanthine oxidase activity has not been studied with respect to the metabolism of high-dose 6MP. We described three patients who developed severe leukopenia due to thiopurine therapy for rheumatoid arthritis and who had very low levels of 5' nucleotidase activity in pMNC [53]. So far, it has been clearly demonstrated in the literature that the levels of TPMT activity have a major influence on the metabolism of 6MP, but other purine enzymes, which have been less well studied, may also interfere with the metabolism of 6MP.

This pilot study is the first which measured TPMT activity in pMNC during the consolidation treatment of ALL and it demonstrated an increase in the levels of MetIMP in pMNC during successive courses of treatment with high-dose 6MP. This pilot study also showed the limitations involved when using pMNC: a high percentage of TPMT assays failed due to leukopenia in the patients. Since leukopenia often occurs and RBC transfusions are frequently required during the consolidation treatment, the influence of high-dose 6MP infusions on TPMT activity can only be investigated further when a TPMT assay that requires less pMNC has been developed.

Acknowledgments

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References

