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Methotrexate Binds to Recombinant Thiopurine S-methyltransferase and Inhibits Enzyme Activity after High-dose Infusions in Childhood Leukaemia

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ABSTRACT

PURPOSE

Important drugs in the treatment of childhood ALL are 6-mercaptopurine (6-MP) and methotrexate (MTX). Thiopurine methyltransferase (TPMT) is a polymorphic enzyme causing variability in 6-MP response and toxicity. During acute lymphoblastic leukaemia (ALL) treatment, we have investigated the fluctuation in TPMT enzyme activity over time, as well as the effect of high-dose MTX infusions on TPMT enzyme activity and 6-MP metabolites.

METHODS

Fifty-three children with ALL treated according to the NOPHO-ALL 2000 protocol were included. TPMT enzyme activity was measured at six different times starting from diagnosis until after the end of maintenance treatment. TPMT and 6-MP metabolites were measured before and 66 hours after start of high-dose MTX infusions. The interaction between MTX and TPMT was investigated in vitro using recombinant TPMT protein and a leukemic cell line.

RESULTS

Forty percent of *TPMT* wild-type individuals had deceptively low TPMT enzyme activity compared to genotype at the time of diagnosis. TPMT decreased significantly 66 hours after the start of high-dose MTX infusions (-9.2 per cent, p=0.013). MTX bound to recombinant TPMT protein and inhibited the TPMT enzyme to a remaining activity of 16 percent.

CONCLUSIONS

This study shows that TPMT genotyping should be preferred in children with ALL, since 40 per cent of *TPMT* wild-type children are at risk of initial under-dosing of 6-MP in cases where

only TPMT enzyme activity is determined. MTX inhibits the TPMT enzyme activity after high-dose MTX infusions due to protein binding.

Introduction

In the Nordic countries, children diagnosed with acute lymphoblastic leukaemia (ALL) follow a common treatment protocol where stratification into risk groups based on prognostic factors determines the exact treatment schedule. Of the about 180 children annually diagnosed in the Nordic Countries, the Event-Free Survival is approximately 80 percent [1]. Treatment-related toxicity can be life-threatening and is the primary cause of less intensive, interrupted or even discontinued maintenance chemotherapy which can lead to an increased risk for relapse [2]. The antimetabolite 6-mercaptopurine (6-MP) is an important part of the ALL treatment and is partly metabolised by the enzyme thiopurine methyltransferase (TPMT).

TPMT activity varies within populations as a result of common genetic polymorphism [3]. Individuals carrying two non-functional *TPMT* alleles (1 out of 300 in Caucasian populations) are at an increased risk for severe side effects if treated with conventional thiopurine doses [4-5]. Approximately 9 percent of the Caucasian population are *TPMT* heterozygous or carrying one non-functional allele. Studies have shown that these individuals also benefit from dose reduction [4]. Since the implementation of the Nordic Society of Paediatric Haematology and Oncology NOPHO ALL-2000 protocol, it is mandatory to determine TPMT status before the start of 6-MP treatment.

In the NOPHO ALL-2000 protocol, MTX is given from the point of diagnosis (usually as intrathecal injections), used as an oral treatment during a maintenance treatment block, as well as in high-dose intravenous infusions starting in the consolidation therapy. For example for children treated according to the standard risk protocol, high-dose-MTX (HD-MTX) is given in a dose of $5g/m^2$ during a time of 24 hours and calcium folinate rescue ($15\ mg/m^2$) from 42 hours after the start of MTX infusion. In the maintenance therapy, MTX is given orally once weekly ($20\ mg/m^2$/kg bodyweight target dose) and 6-MP orally every evening

from 17 weeks until 2 to 2.5 years after diagnosis. The administration of 6-MP and MTX affect S-adenosyl L-methionine (SAM) metabolism in the cell by inhibiting folate-dependent regeneration of S-adenosyl homocysteine (SAH). Inhibition of DHFR by MTX (which thus inhibits formation of tetrahydrofolate) increases plasma concentration of SAH [6]. Both 6-MP and MTX inhibit de novo purine synthesis (DNPS) and the combination of 6-MP and MTX leads to a pronounced DNPS inhibition in vivo, compared to 6-MP or MTX alone [7].The interaction between the drugs is complex and cellular response to the drugs is prompt. In addition, genetic polymorphisms in gene encoding transporters (such as the reduced folate carrier) and enzymes involved in MTX uptake and metabolism affect the treatment outcomes and toxicity [8].

Aim

6-mercaptopurine (6-MP) and methotrexate (MTX) are important drugs in the treatment of childhood ALL. Thiopurine methyltransferase (TPMT) is a polymorphic enzyme causing variability in 6-MP response and toxicity. We investigated the fluctuation in TPMT enzyme activity from time of diagnosis until after the end of maintenance treatment, as well as the effect of high-dose MTX infusions on TPMT enzyme activity and 6-MP metabolites. Recombinant protein was used to investigate binding of MTX to the TPMT protein and a leukaemic cell line to study effects on TPMT enzyme activity in vitro.

Methods

Patients

Following the NOPHO ALL-2000 protocol, fifty-three children with ALL were enrolled in this study. According to the protocol, patients were placed into risk groups depending on pre-therapeutic factors and treated accordingly. Classification criteria of risk groups are listed in Table 1 and use the following protocols: standard intensity, intermediate intensity, intensive, very intensive and extra intensive, respectively. Children following the standard or intermediate intensive protocol received 25 mg /m^2 6-MP per day from day 50 onwards, whereas 60 mg/m^2 per day was given to children included in the intensive, very intensive and extra intensive protocols. During maintenance treatment (day from diagnosis dependent on protocol), standard daily target doses adjusted for TPMT status were as follows: TPMT wild-type: 75 mg/m^2, TPMT heterozygous individuals (50 mg/m^2) and TPMT homozygous defect individuals: 5-10 mg/m^2. MTX was given orally in a target dose of 20 mg/m^2, given once a week as a single dose. During high-dose MTX, the weekly oral MTX was suspended. Doses of 6-MP and MTX were always adjusted to the white blood cell count (target 1.5 to 3.5x 10^9/l), according to the NOPHO ALL-2000 protocol.

Sampling

Blood samples to determine TPMT enzyme activity were obtained at the point of diagnosis, days 15 and 29 (during induction treatment), 106 (beginning or end of consolidation dependent on protocol), at one occasion (approximately 400 days after diagnosis) during the maintenance treatment period, as well as at one occasion 3 to 24 months after cessation of maintenance treatment (Table 2). DNA was isolated to determine *TPMT* genotype from the sample obtained from the point of diagnosis. To determine the effect of HD-MTX infusions on TPMT, thioguanine nucleotides (TGN) and methylthioinosine monophosphate (meTIMP) levels, two separate blood samples were collected either at day 106 or at any time high-dose

MTX infusions were given during maintenance therapy, one sample were taken prior to and one approximately 66 hours after the start of MTX infusion. Calcium folinate rescue were given 42 hours after the start of MTX infusion. Patients received red blood cell transfusions during the study period when needed. This study was approved by the Local Ethics Committee at Linköping University (Ref. no. 01-297). All children and/or their parents gave written consent for their blood samples to be used in research.

TPMT genotyping

DNA was isolated from whole blood using the QIAamp DNA mini kit (Qiagen, Germany, Hilden). Three common variants in the *TPMT* gene (238G>C, 460G>A and 719A>G) were searched for by pyrosequencing as previously described [9].

TPMT enzyme activity assay in red blood cells

TPMT enzyme activity was measured as previously described in Pettersson *et al.* (2002) by determination of the formation of 6-methylmercaptopurine (6-meMP) from 6-mercaptopurine using radio-labelled S-adenosyl-L-methionine as the methyl donor. Product formation was measured using a scintillation counter. One unit of enzyme activity represents the formation of 1 nmol of 6-meMP per millilitre of packed red blood cells (pRBC) per hour of incubation. Cut off points of 9.0 U/ml pRBC and 2.5 U/ml pRBC were used to distinguish high from intermediate enzyme activity and intermediate from low enzyme activity, respectively [10-11].

Determination of TGN and meTIMP

The concentrations of TGN and meTIMP were determined in washed red blood cells using high-performance liquid chromatography (HPLC) as described previously [11]. Results were expressed as pmol/8×10^8 RBC. $^\beta$Normalized metabolite levels were calculated by dividing the measured metabolite levels with daily 6-MP dose..

In vitro incubations of the cell line MOLT4 and MTX

MOLT4 cells, a human T-lymphoblastic cell line from American Tissue Culture Collection (ATCC, Rockville, USA), was cultured in RPMI1640 supplemented with 10 percent foetal calf serum (Gibco, Paisley UK) and sub-cultivated twice every week. Methotrexate (Sigma-Aldrich St Louis, USA) was dissolved in 50 mM NaOH and added to the cell culture (20 ml of $0.3x10^6$ cells/ml) which gave the final concentration of 90 µM and was incubated for 24, 48 and 72 hours at 37 °C in an atmosphere of 5 percent CO_2. Cells incubated only with NaOH in the final concentrations as for the MTX treated cells (0.3 percent) were used as controls (control vehicle, CV). To check that the CV did not significantly influence the TPMT enzyme activity, untreated cells were also included in each experiment. All experiments were performed in triplicates and at each time point two culture flasks with the same treatment were harvested. At the time of harvesting 3.5×10^6 cells were transferred to a 50 ml-tube. The incubation was ended by removing the medium after centrifugation at 500 x g for 10 minutes. The cell pellets were washed in 10 ml PBS, centrifuged, re-suspended in 1 mL PBS and centrifuged again. The cell pellets were stored in -80°C until protein extraction.

At the time for protein extraction, the pellets were placed on ice and sonicated 4 x 5 seconds in 500 µL extraction buffer (50 mM Tris-HCl (pH 7.6), 2 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20 percent glycerol and 0.5 percent Nonidet P40). Samples were vortexed and centrifuged at 30,000 x g at 4°C for 15 minutes. The supernatants were collected and the protein concentration was determined using the DC Protein Assay (Bio-Rad Laboratories, Hercules, California, USA) using a standard curve of bovine serum albumin and measured at 750 nm by a Molecular Devices Versa Max Tunable Microplate Reader and the computer software Soft Max Pro v 5.

TPMT enzyme activity in MOLT4 cells

The TPMT enzyme activity in MOLT4 cells was determined by measuring the rate of formation of 6-methyl-mercaptopurine from 6-MP using ^{14}C-labeled S-adenosyl-L-methionine as the methyl group donor [12]. An amount of 200 µg of the total cellular protein was incubated in duplicates for 2 hours at 37°C in 400 µl of 47 mmol/l potassium phosphate buffer (pH 7.5), supplemented with 3 mmol/l 6-MP, 0.4 mmol/l DTT, 0.2 mmol/l allopurinol, and a mixture of S-adenosyl-L-methionine (SAM) and ^{14}C-labeled SAM (GE Healthcare, Uppsala, Sweden) giving a total SAM concentration of 10 µM. After 2 hours, all reactions were stopped by the addition of 2 ml of acetonitrile. The organic phase was collected, evaporated and then reconstituted in 20 mmol/l KH_2PO_4 (pH 2.7) to total volume of 100 µL. A 50 µl sample was analysed by high performance liquid chromatography using a Purospher STAR, RP18 (3 µm, 55 x 4mm) column maintained at 30°C (MERCK, Darmstadt, Germany) with a Gemini C18 4 x 2mm precolumn from Phenomenex (Torrance, California, USA). The mobile phase consisted of 20 mmol/l KH_2PO_4 (pH 2.7), with 15 percent methanol (v/v). The ultraviolet (UV) signal at 290 nm was used to determine the retention time for meMP and the signal of a scintillation detector was used to determine the enzyme activity. The TPMT enzyme activity of MTX treated cells were expressed as percentage of CV treated cells.

In vitro studies using recombinant TPMT protein

Construction of TPMT variants

Human wild-type TPMT, encoding residues 1–245, was cloned into vector pET28-Lic, generously provided by Structural Genomics Consortium (SGC; Toronto, Canada) and was transformed into the *E. coli* strain BL21-Codon plus (DE3)-RIL (Novagen, Gibbstown, New Jersey, USA) for large-scale expression.

Large-scale expression and purification

E. coli BL21-Codon plus (DE3)-RIL, transformed with the plasmid pET28-Lic, was expressed in LB medium and cultivated at 37°C until OD600 reached 1.0, and then induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) at a decreased temperature of 21°C. After the harvesting and sonication of the cells, purification was performed on Ni-NTA Superflow (Qiagen, Hilden, Germany), the His-tag was removed with biotinylated thrombin (Novagen) and further purified on the Superdex 200 10/30 column (GE Healthcare Biosciences, Uppsala, Sweden). Protein was stored in a 20 mM potassium phosphate buffer (pH 7.3), with 150 mM NaCl, 0.5 mM Tris-2-carboxyethyl phosphine (TCEP) and 10 percent glycerol at -80 °C.

Recombinant TPMT enzyme activity measurements

Spectrophotometry was applied to measure the enzyme activity of recombinant TPMT with 84 μM 6-mercaptopurine as substrate and measured at room temperature on a Hitachi-U-2800 spectrophotometer [13]. The decrease in absorbance was monitored at 320 nm during 30 minutes using a TPMT protein concentration of 1 μM, 1 mM SAM and 200 μM MTX in 20 mM potassium phosphate (pH 7.3), 75 mM NaCl, 2 percent glycerol, 0.5 mM Tris-2-carboxyethyl phosphine (TCEP). The enzyme activity was expressed as a percentage of the activity measured on TPMT protein incubated with CV.

Fluorescence measurements

To determine the binding constant of MTX to TPMT, MTX was titrated in an interval between 0 to 250 μM. Aliquots of 1 μl MTX from a stock solution of 10 mM MTX dissolved in 0.1 M NaOH were added to 1 μM TPMT protein in 20 mM potassium phosphate (pH 7.3), 75 mM NaCl, 2 percent glycerol, and 0.5 mM TCEP. Fluorescence

measurements were performed at 21°C with the excitation wavelength of 295 nm specific for tryptophans and emission spectra recorded at 310-550 nm (Fluoromax-4 from Horiba). The maximum intensity of the Trp–fluorescence obtained at 340 nm was plotted against the increased concentration of MTX.

Statistical analysis

Statistical analysis was conducted using SPSS 19.0 (IBM SPSS Statistics 19) and Graph Pad Prism Software 5.01. For cell line experiments parametric statistics were used (mean and SEM) and differences between cell treatments were analysed using Student's t-test. For analysis of variables among patients, non-parametric statistics were used (median and interquartile range and to compare groups, Wilcoxon's test was used. A p-value <0.05 was considered significant.

Results

A total of 53 children, with T or pre-B-ALL ages 7.5 ± 4.6 years (range 1-17 years) were included in this study (Table 1) and followed the NOPHO ALL-2000 treatment protocol. The majority (81 percent) were treated according to the standard and intermediate intensity protocol, whereas 11.3 percent were treated with the intensive protocol; 3.8 percent with the very intensive and 3.8 percent with the extra intensive protocol. Forty-nine children (92.5 percent) were genotyped as *TPMT*1/*1*, 7.6 percent as heterozygous for one non-functional *TPMT* allele. None of the 53 children were TPMT deficient (TPMT<2.5 U/ml pRBC) or carried two non-functional alleles. Samples of two of the wild type patients were only available at diagnosis and not for follow up.

TPMT enzyme activity during treatment

The median TPMT enzyme activity upon diagnosis was 9.5 U/ml pRBC (interquartile range 7.8 to 10.9) for *TPMT* wild-types (n=47) and 4.7 U/ml pRBC (interquartile range 4.6 to 5.2) for *TPMT* heterozygous individuals (n=4). Of individuals with the *TPMT* genotype *TPMT*1/*1*, 40 percent had an initial enzyme activity at diagnosis below the cut off for normal activity (9 U/ml pRBC). All four heterozygous individuals (*TPMT*1/*3A* or *TPMT*1/*3C*) had an initial enzyme activity below 9 U/ml pRBC. TPMT enzyme activity was measured at 6 different points in time from diagnosis until up to 24 months after the end of maintenance therapy. The TPMT enzyme activity in wild-type individuals increased significantly and reached its highest value at day 106 (median TPMT 14.9 versus 9.5 U/ml pRBC at diagnosis, p=0.001), (Figure 1). The four individuals heterozygous for TPMT also showed an increase in TPMT enzyme activity during the first three months (median TPMT 8.3 versus 4.7 U/ml pRBC), although not statistically significant. The TPMT enzyme activity seemed to stabilise during the maintenance treatment (Figure 1).

In vivo effect of MTX on TPMT and 6-MP metabolites

Samples were obtained at the time for high-dose MTX infusions from those patients when sufficient blood was available for research purposes. TPMT analysis was prioritised over metabolite measurements when a sufficient amount of blood was not available for both assays. During the time period prior to infusion of high-dose MTX and until 66 hours later, The TPMT levels decreased by 9.2 percent (interquartile range -13 to +0.65 percent, n=21, p=0.013), from the beginning of MTX infusion through 66 hours post infusion whereas meTIMP did not change and TGN levels decreased 18 percent (-26 to -15 percent, n=15, p=0.006) (Table 3). The median MTX plasma concentration 23 hours from start of infusion was 84 µM (interquartile range 57-102 µM, n=19).

In vitro effect of MTX on TPMT enzyme activity in MOLT4 cells

The TPMT enzyme activity in MOLT4 cells showed an initial increase after 24 hours of incubation with 90 µM MTX. However, a significant decrease to 26 percent of CV was observed after 48 hours, and the enzyme activity was only 1.8 percent after 72 hours (Figure 3). The CV did not affect TPMT enzyme activity as shown from comparisons with untreated MOLT4 cells in Figure 2.

In vitro effect of MTX on TPMT enzyme activity of the recombinant protein

The hydrophobic active site of TPMT (Figure 3A) harbours three out of the five tryptophans in the structure of TPMT (Figure 3B). To determine whether MTX is a potential ligand to TPMT, tryptophan fluorescence (with titration of MTX to TPMT) was performed. Figure 4 shows that the binding profile of MTX to TPMT and the K_d of approximately 24 µM could be determined. The remaining enzyme activity of the recombinant wild-type TPMT protein after incubation was 16 percent with 200 µM MTX.

Discussion

In this study it has been shown that TPMT enzyme activity fluctuated from time of diagnosis throughout the time of treatment. At time of diagnosis, 40 percent of *TPMT* wild-type ALL patients displayed a deceptively low TPMT enzyme activity compared to genotype (Figure 1). *TPMT* heterozygous individuals had a TPMT enzyme activity within the range of our reference values (2.5-8.9 U/ml pRBC). In *TPMT* wild-types, TPMT enzyme activity increased from start of treatment, at least until day 106, when all *TPMT* wild-type individuals showed a TPMT enzyme activity above 9 U/ml pRBC. Even though not specifically investigated in our study, the RBCs do not constitute a homogenous group and the RBCs have a life span of 120 days. Lennard *et al.* (2001) showed that the RBC population at diagnosis was influenced by the disturbed haematopoiesis and the population consisted of an excess of old RBCs [14]. In that study, a density gradient centrifugation of the red blood cells showed that the population corresponding to the older cells displayed a lower TPMT enzyme activity compared to newer cells. In our study, the TPMT enzyme activity increased the first three months, in accordance with the theory that the increase in enzyme activity reflects the recovering haematopoiesis in the patients. However, other drugs could affect TPMT enzyme activity although it is difficult to say what impact these drugs may have on the enzyme activity compared to the RBC age. Also, RBC transfusion may have an impact on the TPMT activity, although Brouwer et al., (2005) showed that this effect was not significant for wild type individuals receiving transfusions [15]. Determining TPMT enzyme activity at diagnosis may lead to a deceptively low TPMT value which could then lead to the risk to initially under-treating the patient, particularly if this result is used to determine the starting dose of 6-MP. Preferably, TPMT genotyping should be performed using techniques that allow for the detection of novel/rare variants, however, most laboratories only genotype for the three most common TPMT sequence variants. Our results

is in accordance with a recently published paper in a large cohort of ALL patients in UK [16].

In vivo and in vitro effects of MTX on TPMT and 6-MP metabolites

When measuring TPMT enzyme activity, TGN and meTIMP levels before and after high-dose MTX infusions in children already on oral 6-MP treatment, we found a median decrease in TPMT enzyme activity of 9.2 percent 66 hours after start of MTX infusion (Table 3). Despite the decrease in enzyme activity, we did not see any effect on meTIMP levels at the same time point. TGN levels decreased which is a result in accordance with previous studies [17-18]. Zaza *et al.* (2005) investigated gene expression and TGN levels after high-and low dose MTX infusions in leukemic blasts and showed that patients treated with 6-MP alone achieved higher TGN concentrations than those treated with the combination of 6-MP and MTX. Furthermore, there were distinct sets of upregulated genes in each treatment group [18]. We hypothesise that the interaction between the TPMT protein and MTX is prompt, however, the effect of this inhibition on the metabolism in the context of TGN and meTIMP formation may be a phenomenon seen at a later time point. It could also be that other mechanisms such as those discussed above, will mask the effect of TPMT inhibition on drug metabolism.

A study by de Beaumais *et al.* (2011), which aimed to examine metabolite levels during treatment, discovered that TGN levels were dependent on *TPMT* genotype as well as the patient's age, whereas meTIMP was dependent on *TPMT* and *ITPA* genotypes [19]. ITPase deficiency has been shown as a risk factor for 6-MP toxicity [2].

In this study, the metabolite levels were only measured prior to and after one occasion of MTX infusions. The effect of *ITPA* genotype on metabolite levels during treatment was a subject that the authors felt went beyond the scope of this article.

To the knowledge of the authors, no study has yet investigated the interaction between 6-MP and MTX by focussing specifically on the influence of MTX on TPMT enzyme activity. The decrease in TPMT enzyme activity was intriguing, and by using recombinant TPMT protein and biophysical characterisation studies of TPMT were carried out when incubated with MTX in vitro. The hypothesis was that MTX was a potent ligand to TPMT due to effects on TPMT enzyme activity after high-dose MTX infusions in children with ALL. The determined crystal structure of the three-dimensional human TPMT [20] showed an active site (Figure 3A) with a large hydrophobic cavity possible of binding purine analogues as well as other hydrophobic compounds. Experiments in our laboratory have shown that, for example, the hydrophobic compound anilino-naphtalene sulfonate (ANS) binds to TPMT with high affinity (data not shown). The protein sequence of TPMT contains five tryptophans, three of them situated in or near the active site (Figure 3B). A method that used fluorescence to study the quenching of tryptophans when adding MTX was used in this study in order to investigate potential binding. The titration of MTX to recombinant TPMT protein (Figure 4) showed binding with an approximate K_d of 24 µM. Taking into account that the median plasma concentration of MTX after 23 hours of infusion was 84 µM, the conclusion that has been drawn is that MTX binds to TPMT at relevant physiological concentrations. A leukemic cell line (MOLT4) was also used and the authors also investigated the effect of 90 µM MTX on the TPMT enzyme activity after 24, 48 and 72 hours of incubation. During the first 24 hours of incubation, an increase in TPMT enzyme activity was detected; however, after 48 hours a significant decrease was found in enzyme activity compared to CV (26 percent of CV, $p < 0.001$), an inhibition that continued over time; after 72 hours the enzyme activity was almost undetectable. We cannot rule out the

possibility that the decrease in TPMT activity partly is reflecting an antiproliferative effect of MTX, even though our activity measurements as always done in the same number of cells. It is not clear as to why the enzyme activity increased at the beginning of MTX incubation. A study by Brouwer *et al.* 2005 [15], showed that TPMT enzyme activity increased in MOLT4 cells after incubation with MTX, however, in their study, cells were simultaneously treated with methionine, thus creating increased amounts of SAM in the cells. As SAM stabilises TPMT it could be that the increase in enzyme activity is due to SAM, not to MTX [21].

Conclusion

In childhood ALL, the TPMT status should be determined by using TPMT genotyping. Genotyping is preferable as enzyme activity measurement during the first 106 days of treatment will lead to a misclassification. This could potentially lead to initial under-dosing of these individuals. Preferably, the genotyping technique should allow for detection of novel variants. MTX binds to TPMT in vitro and inhibits the enzyme activity both in vitro and in children with ALL after high-dose MTX infusions. Understanding the mechanisms of drug synergism as well as other causes for differences in drug response is important in order to improve treatment and decrease treatment-related toxicity.

Competing Interests

There are no competing interests to declare.

Acknowledgements

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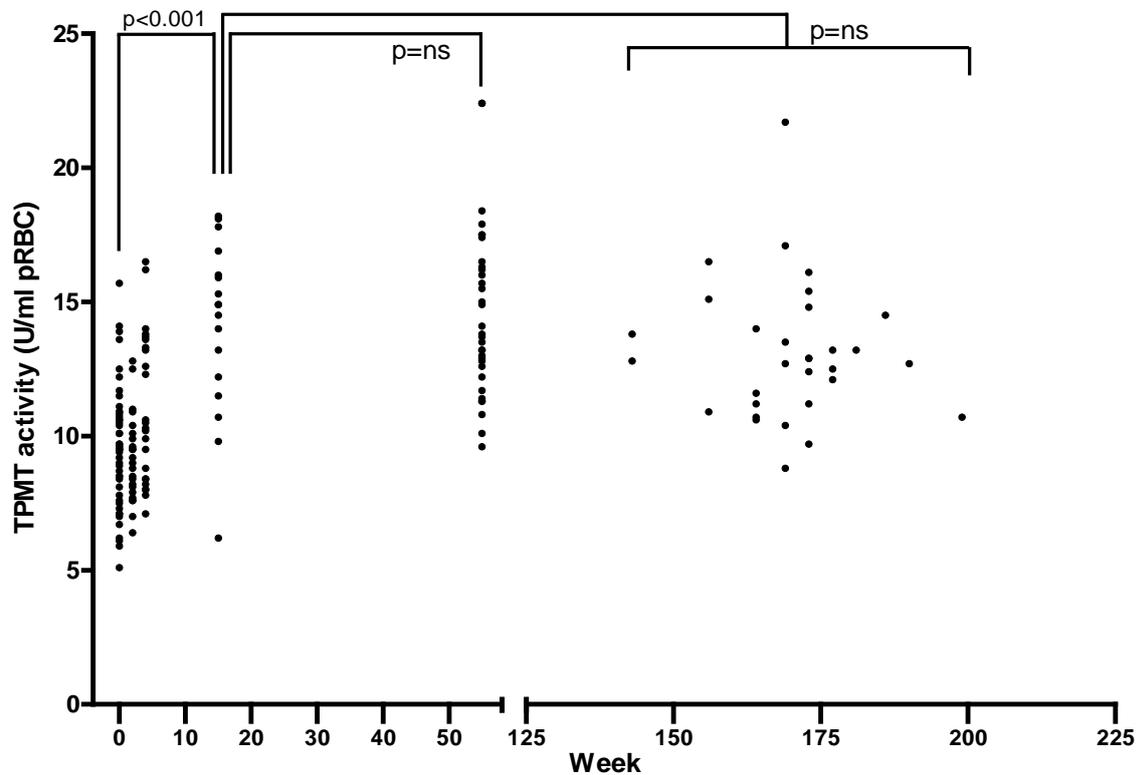
FIGURE LEGENDS

Figure 1. TPMT activity in 47 TPMT wild-type children with ALL from diagnosis until up to

2 years after cessation of maintenance therapy. Patients were sampled at 5 occasions during

treatment protocol, and one time from 3 months up to 24 months after cessation of therapy.

ns= non-significant differences between time points. TPMT activity increased the first 18
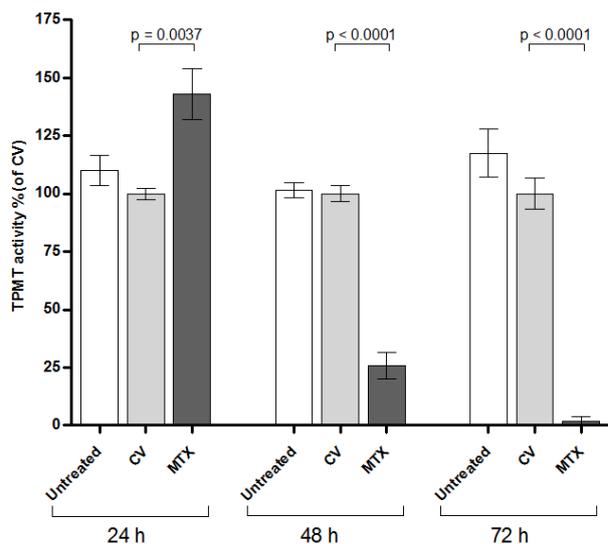
weeks of treatment.

Figure 2. TPMT activity in MOLT4 cells after 24, 48 and 72 h of incubation with methotrexate (MTX, final concentration 90 µM). Results are shown as mean percentage of respective control vehicle (CV) ± SEM (N=6). The TPMT activity in untreated cells compared to cells treated with CV is also shown. TPMT activity was increased the first 24 h of incubation. At 48 h, the TPMT activity was only 26 % of CV treated cells, and the activity was only 1.8 % after 72 h.
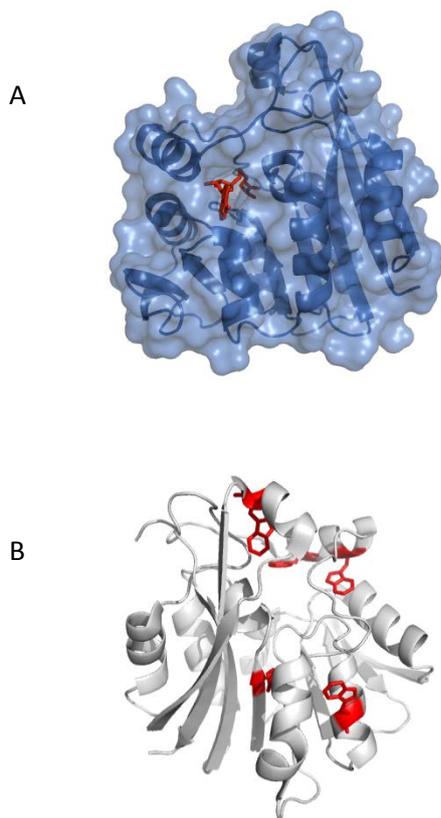
A

B

Figure 3. A) Three-dimensional structure of human TPMT illustrating the active site with the co product S-adenosyl L- homocysteine (PDB code 2BZG) in red. B) Three-dimensional structure of human TPMT illustrating tryptophan positions (red), (PDB code 2BZG)
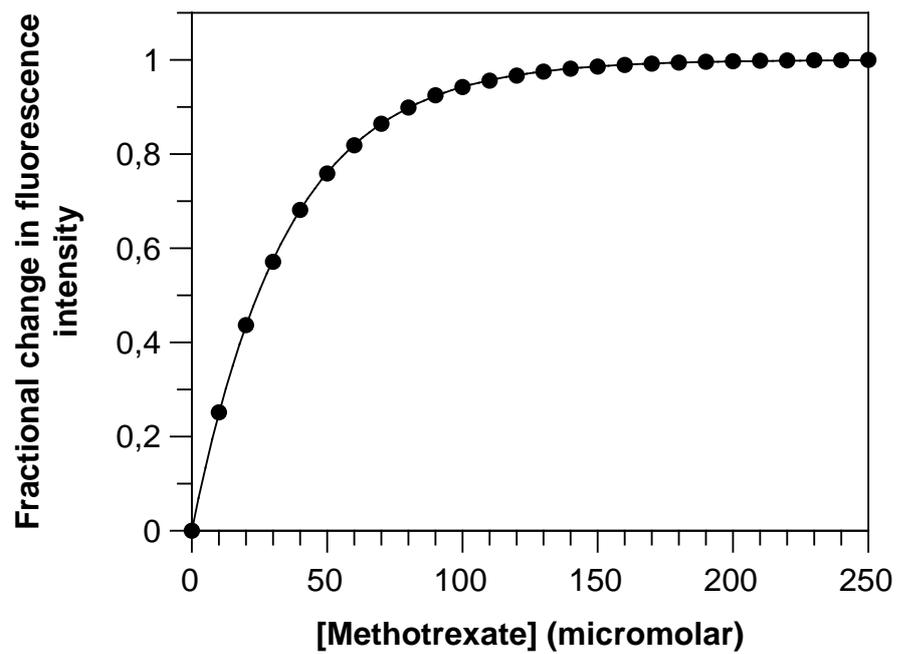
Figure 4. Titration of 0 to 250 µM methotrexate to human recombinant TPMT as monitored

by tryptophan fluorescence intensity at 340 nm.

Table 1. Patient Characteristics

Diagnosis	Pre-B-ALL	49 (92.5 %)
	T-ALL	4 (7.5 %)
ALL-2000 protocol	Extra Intensive (EI)	2 (3.8 %)
	Very intensive (VI)	2 (3.8 %)
	Intensive (I)	6 (11.3 %)
	Intermediate Intensive (II)	23 (43.4 %)
	Standard Intensive (SI)	20 (37.7 %)
Gender	Male	24 (45.3%)
	Female	29 (54.7%)
Age	Median year (Range)	7.5 (1-17)
TPMT genotype	*TPMT*1/*1*	49 (92.5 %)
	*TPMT*1/*3A*	3 (5.7 %)
	*TPMT*1/*3C*	1 (1.9%)

Factors stratifying patients to the respective therapy group:

EI; WBC > 200.1 x 10^9/l, very slow response (Day 29: M3)

VI; Age >5 years, WBC 100.1 – 200 x 10^9/l, > 5 years, CNS-ALL, >5 years, T-cell ALL with mediastinal mass.

I; WBC ≤100 x 10^9/l, rest of the patients according to high risk criteria.

II; Age 1 to <10 years and WBC 10.1 - ≤50.0 x 10^9/l or Age ≥10 years and WBC ≤ 50.0 x 10^9/l. No high risk criteria.

HR-criteria; WBC, 11q23/MLL- rearrangements, t(9;22)(q34;q11)/BCR-ABL,

t(1;19)(q23;p13)/E2A-PBX1, Hypodiploidy (<45 chromosomes), T-cell ALL, CNS-ALL,

Testis ALL, Poor response (BM Day 15: M3 and/or BM Day 29: M2 or M3).

SI; Age, 1 to <10 years and WBC≤10.0 x 10^9/l, No high risk criteria

Table 2. Flowchart of available blood samples during study

TPMT genotype	At diagnosis	Day 15	Day 29	Day 106	Day~400 (during the maintenance treatment)	Follow up (>3 months after end of treatment)
All patients	51 (53 $^\beta$)	27	27	20	36	35
TPMT wild type	47 (49$^\beta$)	23	24	17	33	33
TPMT heterozygous	4	4	3	3	3	2

$^\beta$ Two TPMT wild type individuals were not available for follow up samples.

Table 3

Median (interquartile range) pre-HDMTX infusion TPMT (U/ml pRBC), RBC TGN and

meTIMP levels (pmol/8x10^8 RBC) with percentage change around 66 hours after initiation of

HD-MTX.

	0 hours (pre-infusion)	~66 hours % change	Change versus pre-infusion values
TPMT (U/ml pRBC)	13.4 (11.4-16.2)	-9.2 (-13.0 to + 0.65)	P=0.013 N=21
TGN (pmol/8x10^8 RBC)	111.5^β (79-271)	-18.1 % (-26 to -14.6)	P=0.006 N=15
meTIMP (pmol/8x10^8 RBC)	1609^β (427-3900)	0 % (-20.5 to + 36)	P= 0.917 N=15

^{β}Normalized metabolite levels were calculated by dividing the metabolite levels with daily

6-MP dose. TPMT: Thiopurine methyltransferase; TGN: Thioguanine nucleotides;

meTIMP: methylthioinosine monophosphate.