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Post-translational stabilization of thiopurine S-methyltransferase by S-adenosyl-L-methionine reveals regulation of TPMT*1 and *3C allozymes☆

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ABSTRACT

Thiopurine S-methyltransferase (TPMT; EC 2.1.1.67) plays a pivotal role in thiopurine treatment outcomes. However, little has been known about its intracellular regulation. Here, we describe the effect of fluctuations in physiological levels of S-adenosyl-L-methionine (SAM) and related metabolites on TPMT activity levels in cell lines and erythrocytes from healthy donors. We determined higher TPMT activity in wild-type TPMT*1/*1 individuals with high SAM concentrations ($n = 96$) compared to the low SAM level group ($n = 19$; $P < 0.001$). These findings confirm the results of our in vitro studies, which demonstrated that the restriction of L-methionine (Met) in cell growth media reversibly decreased TPMT activity and protein levels. Selective inhibition of distinct components of Met metabolism was used to demonstrate that SAM is implicitly responsible for direct post-translational TPMT stabilization. The greatest effect of SAM-mediated TPMT stabilization was observed in the case of wild-type TPMT*1 and variant *3C allozymes. In addition to TPMT genotyping, SAM may serve as an important biochemical marker in individualization of thiopurine therapy.

1. Introduction

Thiopurine S-methyltransferase (TPMT; EC 2.1.1.67) is a genetically polymorphic enzyme that deactivates thiopurine drugs such as 6-mercaptopurine (6-MP) [1]. Impact of TPMT genetic polymorphisms on pharmacokinetics and toxicity of thiopurines is evident in acute lymphoblastic leukemia (ALL) patients who are homozygous or heterozygous for variant TPMT alleles (TPMT*2, *3A, *3C) [2–4]. They exhibit decreased TPMT activity which correlates with high cytotoxic thioguanine nucleotide (TGN) levels, resulting in high risk for life-threatening myelosuppression if standard doses of thiopurines are administered [5–8]. Homozygous ALL patients with low or absent TPMT activity require a reduction to 10% of the standard dose, whereas dose reduction in heterozygous individuals with intermediate activity is less clearly defined, ranging from 30% to 70% [4].

Although relatively high TPMT genotype-to-phenotype correlations have been observed in many studies [9–12], substantial influence of non-genetic factors on TPMT activity is recognized [13,14]. In genotype-to-phenotype correlation studies, homozygous individuals for wild-type TPMT alleles are normally classed into a single phenotypic group of high methylators, even though they exhibit a wide range of TPMT activity [7,9]. Recently, several studies have shown that factors other than TPMT genotype influence TPMT protein expression and activity [14–19], indicating that novel relevant factors governing high TPMT activity remain to be identified. In addition, low genotype-to-phenotype correlation is most commonly observed in heterozygous individuals with variable intermediate activity [20–22]. Therefore, the identification and understanding of novel factors influencing TPMT activity is crucial for the improvement of efficacy and safety of thiopurine treatment.

Non-synonymous amino acid substitutions in variant TPMT allozymes destabilize the protein structure and increase its susceptibility to proteasome and/or autophagic degradation [23,24]. A few reports have speculated that the binding of S-adenosyl-L-methionine (SAM), the principal cellular methyl donor and TPMT co-substrate, stabilizes the protein structure against in vitro degradation [25–27], and that this interaction has potential clinical relevance [15,16,18].

In mammalian cells, SAM is synthesized from L-methionine (Met) in a reaction catalyzed by methionine adenosyltransferase (MAT). As a SAM-dependent methyltransferase, TPMT is closely linked to the Met cycle, the folate pathway and related processes

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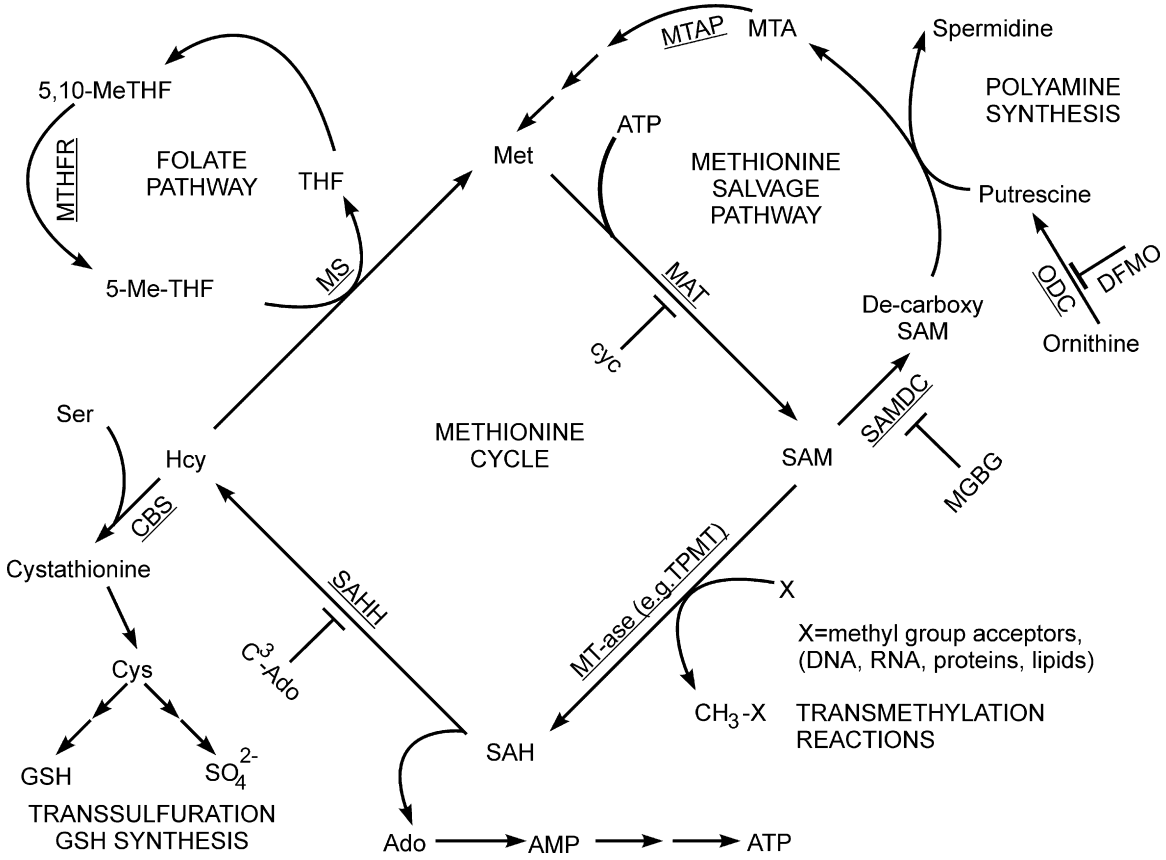

Fig. 1. SAM metabolism, the methionine cycle and related pathways in the liver. Specific enzyme inhibitors used in this study are indicated. Ado, adenosine; C^3-Ado: 3-deazaadenosine; CBS, cystathionine β-synthase; Cyc: cycloleucine; Cys, cysteine; DFMO: α-difluoromethylornithine; GSH, glutathione; Hcy, homocysteine; MAT, methionine adenosyltransferase; Met, L-methionine; 5-Me-THF, 5-methyltetrahydrofolate; 5,10-Me-THF, 5,10-methylenetetrahydrofolate; MGBG: methylglyoxal-bis(guanylhydrazone); MS, methionine synthase; MTA, 5′-methylthioadenosine; MTAP, 5′-methylthioadenosine phosphorylase; MTHFR, 5,10-methylenetetrahydrofolate reductase; ODC, ornithine decarboxylase; SAH, S-adenosyl-L-homocysteine; SAHH, S-adenosyl-L-homocysteine hydrolase; SAM, S-adenosyl-L-methionine; SAMDC, SAM decarboxylase; Ser, serine; THF, tetrahydrofolate; TPMT, thiopurine S-methyltransferase.

(Fig. 1) [28,29]. In this context, our study aimed to identify additional physiological factors, which regulate TPMT activity and impact thiopurine drug response. We demonstrate for the first time that SAM is under physiological conditions implicitly responsible for in vivo TPMT stabilization. Furthermore, TPMT*1 and *3C allozymes were confirmed to be post-translationally stabilized by SAM in several cell lines and primary cells, and a high correlation between TPMT activity and SAM concentration was observed in erythrocytes of healthy individuals. On the basis of these findings we propose that SAM may serve as an important biochemical marker to predict TPMT activity. In addition, SAM may be a promising agent to acutely regulate TPMT activity in order to rapidly decrease excess thiopurine toxicity.

2. Materials and methods

2.1. Study participants, preparation of hemolysates and genotyping assays

This study was approved by the Research Ethics Committee (University of Tartu, Estonia) and included analyses of human samples stored at the Estonian Genome Center (University of Tartu). Informed consent was obtained from all participants of the study. Venous blood (50 ml) from unrelated Caucasian healthy individuals (n = 680) was drawn into K_3-EDTA-treated containers and processed at less than 12 h after collection. DNA isolation was carried out using the conventional salting out procedure [30] and genotyping of TPMT activity deficient TPMT*3B (460G > A, rs1800460) and *3C (719A > G, rs1142345) alleles was carried out by Taqman Genotyping Assays (Applied Biosystems, Foster City, CA) as described previously [17]. Hemolysates (n = 159) were prepared according to the previously described procedure [31,32]. An aliquot (0.1 ml) was used for the routine hemoglobin measurement on a Coulter Ac·T Diff analyzer (Beckman Coulter, Brea, CA).

2.2. SAM concentration and TPMT activity

TPMT activity in cell culture samples and hemolysates was determined as described [18,32]. For SAM measurements in cultured cells, cells were trypsinized, washed twice in PBS and lysed in 0.5 M perchloric acid (100 μl, Sigma, St. Louis, MO) for 20 min on ice. After centrifugation (14,000 × g, 15 min, 4 °C), supernatants were stored at −80 °C until HPLC analysis. For SAM measurements in hemolysates, aliquots (0.2 ml) were thawed only once and precipitated by adding 70% perchloric acid (9 μl), followed by centrifuging twice (14,000 × g, 15 min, 4 °C). SAM was measured immediately in the supernatants by the modified reversed-phase HPLC method as described [33].

Recovery of all analyzed metabolites was evaluated by spiking known amounts of standards into biological samples and was found to be higher than 95% for all analyses. Average value of two independent determinations was reported for all hemolysate analyses.

2.3. Cell culture conditions, transfection, TPMT protein and mRNA expression, statistical analyses and cut-off point determination

Additional materials and methods are described in Supplementary Methods online.

3. Results

3.1. Exogenous Met and SAM result in increase of TPMT activity

We examined the relevance of SAM for the post-translational stabilization of TPMT at the cellular level under physiological conditions. Since SAM is synthesized in the Met cycle (Fig. 1), its intracellular concentration depends on Met and downstream metabolites. We thus evaluated their effect on TPMT activity by modifying the availability of Met in growth media to simulate both the physiologically high (100–200 μM) and low Met levels (<5 μM) [34,35] in embryonic kidney HEK293 and hepatocarcinoma HepG2 cells since TPMT expression was reported to be highly expressed in kidneys and liver [3].

TPMT activity measurements in lysates of kidney HEK293 cells grown in the presence (Met+) or absence (Met−) of Met showed that TPMT activity is rapidly decreased in media lacking Met (Fig. 2A). Met− cells (48 h without Met), when supplemented with Met or its metabolite SAM, showed a significant increase of TPMT activity levels ≥8 h post addition. Due to a significant decrease in viability (data not shown), experiments involving long-term incubation in the absence of Met or SAM (72 h or more) were not performed. To exclude the possible effects of fluctuations of other amino acids or nutrients on the observed increase of TPMT activity, Met-depleted cells were incubated for a short time period in PBS containing Met or SAM, and were found to have a significantly higher TPMT activity than cells incubated in PBS only (Fig. 2B). Increase of TPMT activity was thus the consequence of a specific response to the addition of Met or SAM.

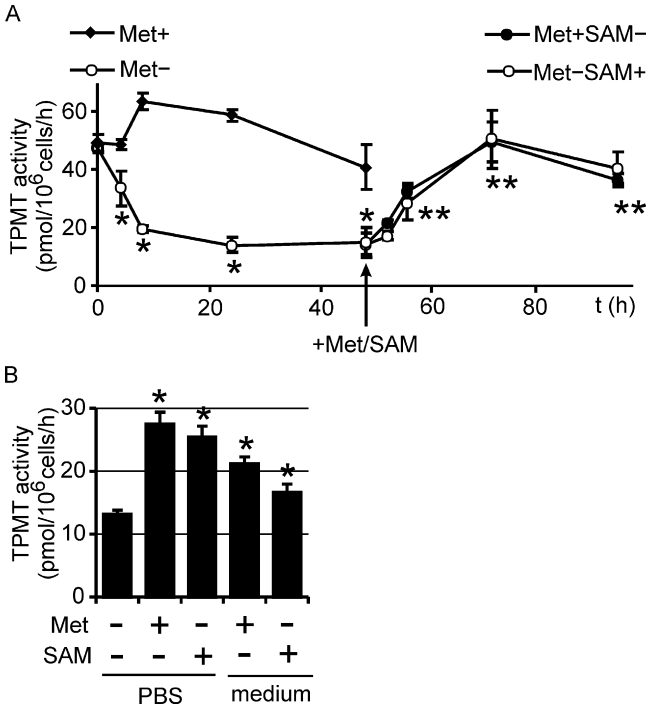

Fig. 2. TPMT activity depends on L-methionine (Met) availability. (A) HEK293 cells were incubated in Met+ or Met− medium for different time periods. After 48 h of growth in Met− medium, 200 μM Met or 500 μM SAM was added (arrow). After the indicated time periods TPMT activity was determined by RP-HPLC. Significant differences up to 48 h of Met depletion (*$P < 0.05$ vs. Met+) and after the addition of Met or SAM (**$P < 0.05$ vs. Met− 48 h) are denoted. (B) Met− HEK293 cells (grown 48 h without Met) were incubated for 4 h in PBS or Met− media containing either 200 μM Met or 500 μM SAM (*$P < 0.05$ vs. PBS only cells).

3.2. Post-translational stabilization of TPMT by intracellular Met metabolites decreases its susceptibility to proteasome degradation

Next we examined whether Met- or SAM-dependent modulation of TPMT activity could be detected in diverse cell types, and whether modulated TPMT activity was reflected in differences in TPMT mRNA and protein levels. Following a 24-h incubation in Met− media, TPMT activity was significantly decreased in HepG2 (Fig. 3A), HeLa and MOLT cells, as well as in freshly isolated human peripheral blood mononuclear cells (PBMC) (Supplementary Fig. S1A), but could be restored by addition of Met. This effect could be mimicked by two Met metabolites, SAM and 5′-methylthioadenosine (MTA). Reversible regulation of TPMT activity by Met metabolites was also observed at the protein level. TPMT immunoblotting showed that in HepG2 (Fig. 3B), HeLa, MOLT (Supplementary Fig. S1B), and HEK293 (not shown) total cell lysates, protein levels correlated well with TPMT activity measurements (Figs. 3A and S1A). However, differences in protein expression were not reflected at the transcriptional level, since TPMT mRNA remained unchanged after 24 h of incubation (Supplementary Fig. S2). Since TPMT phenotypic responses were comparable in all cell lines, further experiments were performed on HepG2 cells, which showed the highest level of endogenous TPMT expression and activity.

To determine whether SAM-dependent modulation of TPMT occurs at the translational or post-translational level, we pre-incubated Met-depleted HepG2 cells with cycloheximide (CHX), a general inhibitor of protein synthesis, followed by incubation with Met, SAM, or MTA (Fig. 3C). TPMT activity was slightly, but not significantly ($P = 0.155$), lower in lysates of Met-, SAM-, or MTA-treated cells pre-incubated with CHX than in controls with no CHX pre-incubation. Therefore, higher TPMT activity and protein levels were observed due to post-translational stabilization of the enzyme. We also checked whether Met restriction, or supplementation with Met, SAM, or MTA, results in significant modulation of intracellular SAM concentration. SAM measurements in cell extracts showed that significantly lower SAM levels were present in Met-depleted cells than in cells incubated with Met or its metabolites (Fig. 3D). In addition, a highly significant correlation between SAM values and HepG2 TPMT activity was observed (Spearman $r = 0.701$; $P = 0.0012$). Altogether, these results provide evidence for acute stabilization of TPMT by SAM via a post-translational mechanism that is present in tumor-derived, transformed and primary cells. Increase of TPMT activity is a consequence of SAM-mediated stabilization of TPMT, reflected in higher protein but not mRNA levels.

TPMT variant allozymes containing non-synonymous amino acid substitutions were shown to be more susceptible to aggregation, polyubiquitinylation and subsequent proteasome degradation, than wild-type TPMT in cells grown under conditions of high Met [27,36]. Since decreased TPMT protein levels were detected in SAM-depleted cells, we attempted to delineate the mode of protein degradation. We used immunostaining and fluorescence microscopy to examine whether degradation of wild-type TPMT via the proteasome pathway is more rapid in SAM-depleted HeLa cells. In the presence of MG132, a protesome inhibitor, we observed that TPMT was present in aggregates in Met− but not in Met+ cells, indicating that high Met metabolite concentrations prevent aggregation and subsequent proteasome degradation of TPMT, possibly by stabilizing the protein structure and increasing its resistance to aggregation and proteolysis (Supplementary Fig. S3).

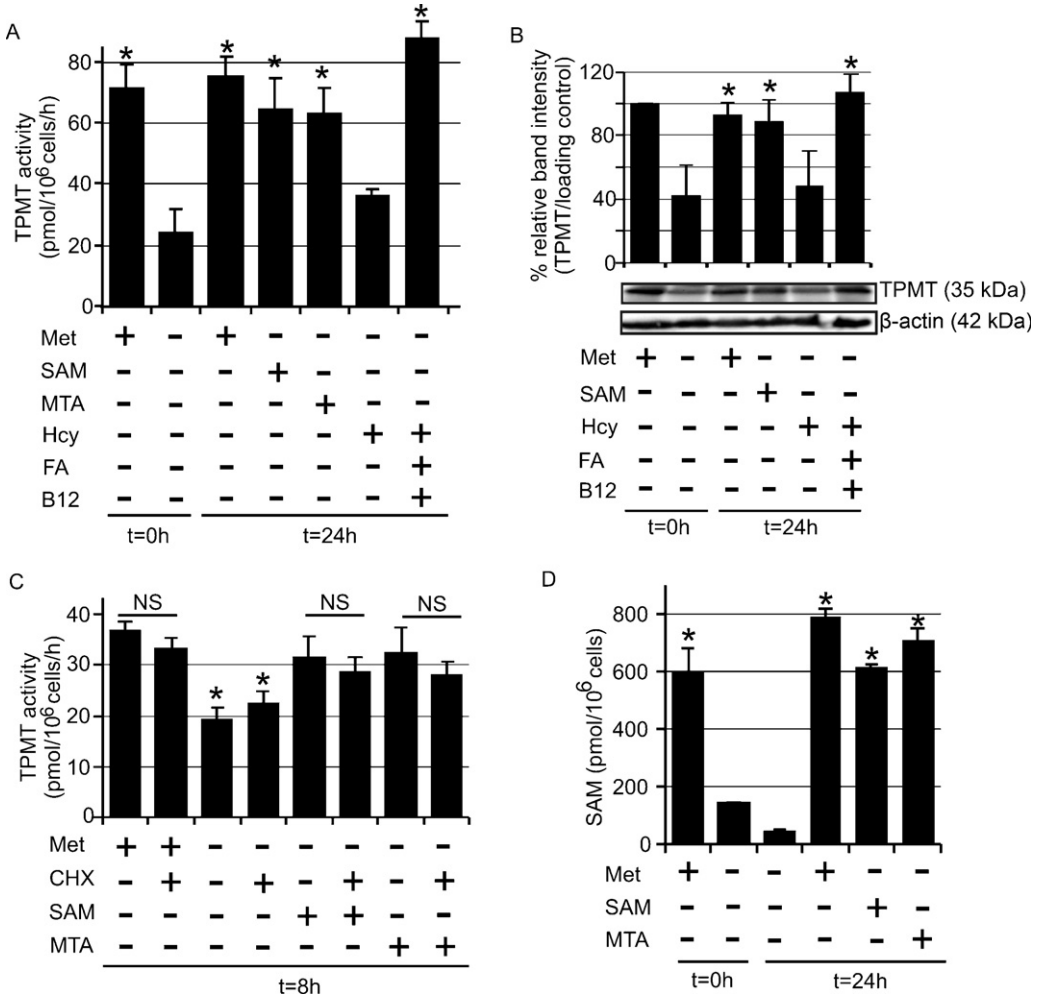

Fig. 3. Met cycle and folate metabolites post-translationally control TPMT activity and protein levels. (A) HepG2 cells were incubated in Met+ or Met− medium for 24 h (t = 0 h). Met (200 μM), SAM (1.5 mM), MTA (0.5 mM), homocysteine (Hcy, 200 μM), cyanocobalamine (B12, 15 μM) and/or folinic acid (FA, 100 μM) were then added to Met− cells and grown for another 24 h (t = 24 h). Differences in TPMT activity levels were followed by RP-HPLC (*$P < 0.05$ vs. Met− cells). (B) TPMT protein levels in total lysates of cells grown under the conditions described in (A) were determined by immunoblotting. TPMT band intensities were normalized to the β-actin band intensity and expressed as percent of control (Met+ t = 0 h) (*$P < 0.05$ vs. Met− cells). Representative immunoblot (lower panel) is shown below the graph (upper panel). (C) HepG2 cells were grown in Met− medium for 24 h (t = 0 h), pre-incubated with 100 μM CHX for 5 min, followed by incubation of cells with Met, SAM, or MTA-containing media for 8 h. Differences in TPMT activity were then determined by RP-HPLC (*$P < 0.05$ vs. Met+; NS, not significant). (D) Cells were grown as described in (A) and intracellular SAM levels were determined in perchloric acid HepG2 cell extracts by RP-HPLC (*$P < 0.05$ vs. Met− 0 h).

3.3. High folate supply enables TPMT stabilization when homocysteine levels are high

To examine whether folate pathway metabolites can indirectly modulate TPMT – since they are precursors of Met, SAM and downstream metabolites – Met-depleted HepG2 cells were incubated with folinic acid (FA), a cell-permeable precursor of 5-methyltetrahydrofolate (5-MeTHF), cyanocobalamine (B12), a co-factor of methionine synthase, and homocysteine (Hcy). Simultaneous treatment with Hcy, FA, and B12 mimicked the Met-dependent increase of TPMT activity and protein levels (Fig. 3A and B). Incubation with Hcy, FA or B12 alone, or in pairwise combinations (not shown), did not result in an increase of TPMT levels. Therefore, sufficient metabolic flux from the folate pathway resulted in an increase of TPMT activity and protein levels only if intracellular Hcy levels were high.

3.4. SAM is responsible for direct stabilization of TPMT

Even though we used several exogenous Met metabolites to modulate intracellular TPMT activity in the experiments described above, this approach did not ensure differential intracellular metabolite levels since Met, SAM, MTA and FA are rapidly converted by enzymes upon entering the cell. To determine if intracellular TPMT levels are stabilized by a specific Met metabolite, selective enzyme inhibitors were used. Since they were found to be less effective after 24 h of incubation (data not shown), and due to the fact that only 1.5- to 2-fold higher TPMT protein levels were observed after short incubation times (4, 8 h), we used activity measurements to accurately quantify TPMT stabilization in these experiments (Fig. 4).

Inhibition of methionine adenosyltransferase by cycloleucine (cyc) prevented SAM synthesis from Met and maintained intracellular SAM at low levels if Met or MTA were added (Fig. 1) [37,38]. In our experiments, pre-incubation of Met− cells with cyc completely prevented an increase of TPMT activity on Met or MTA supplementation, but had no effect when cells were incubated with SAM (Fig. 4A). Thus, high levels of Met or MTA are not themselves sufficient but must be converted to SAM to ensure TPMT stabilization.

In another experiment, α-difluoromethylornithine (DFMO) and methylglyoxal-bis(guanylhydrazone) (MGBG) were used to inhibit

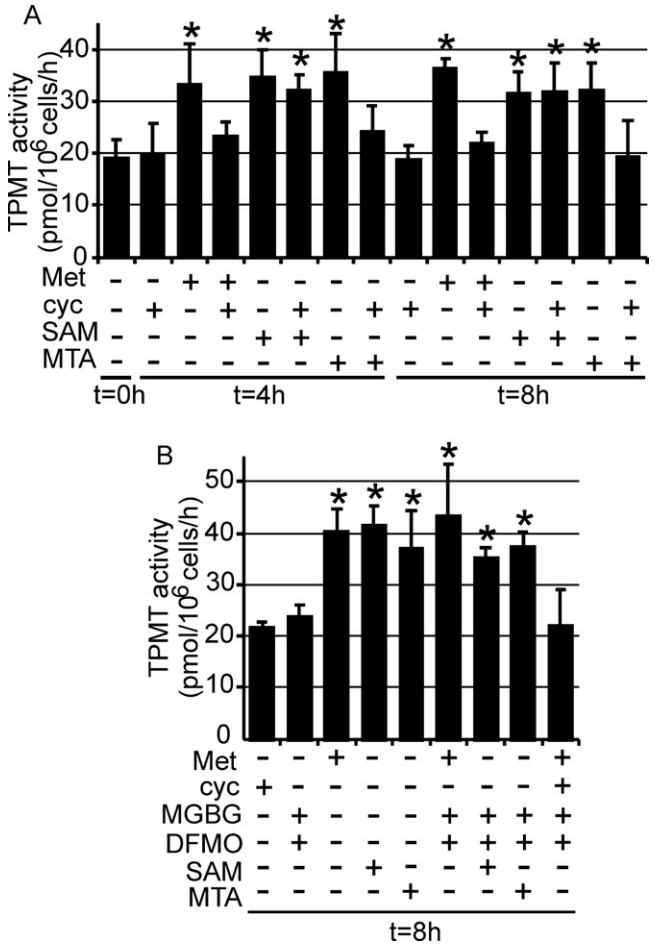

Fig. 4. SAM is responsible for direct stabilization of TPMT. To deplete Met and its metabolites, HepG2 cells were grown in Met− media for 24 h ($t = 0$ h). They were then pre-incubated with inhibitors (see below), and Met (200 μM), SAM (1.5 mM), or MTA (0.5 mM) was added. TPMT activity was followed by HPLC after 4 or 8 h of incubation. (A) Met− cells were pretreated for 1.5 h with 20 mM cycloleucine (cyc), followed by addition of Met, SAM or MTA and measurement of TPMT activity (*$P < 0.05$ vs. Met− $t = 0$ h). (B) Met− cells were pretreated for 1.5 h with both methylglyoxal-bis(guanylhydrazone) (MGBG, 5 μM) and α-difluoromethylornithine (DFMO, 1 mM), followed by incubation with Met, SAM or MTA and TPMT activity determination (*$P < 0.05$ vs. Met− MGBG+ DFMO+ cells).

ornithine decarboxylase (ODC) and S-adenosyl-L-methionine decarboxylase (SAMDC), respectively, in order to prevent SAM decarboxylation and further conversion to MTA (Fig. 1). We ensured high intracellular SAM concentrations and at the same time low levels of downstream SAM metabolites leading to MTA re-synthesis by the addition of Met, SAM, or MTA to cells pretreated with both inhibitors, and found that TPMT activity was higher than in Met− cells (Fig. 4B). In this experiment we included additional control cells, which were pretreated with cyc, DFMO and MGBG before the addition of Met to inhibit also the MAT-catalyzed conversion of Met to SAM, and this prevented an increase of TPMT activity compared to Met− cells. Therefore, we concluded that further conversion of SAM via the Met salvage pathway is not a requirement for TPMT stabilization.

By using a general transmethylation inhibitor we also found that transmethylation reactions and metabolites downstream of SAM in the Met cycle are not involved in the modulation of TPMT activity (Supplementary Fig. S4). In summary, we showed by a pharmacological approach that SAM is the metabolite responsible for direct post-translational stabilization of TPMT.

3.5. SAM depletion results in decreased protein and activity levels of recombinant TPMT*1 and *3C allozymes

To elucidate the effect of intracellular SAM levels on the stability of TPMT variant allozymes, we transiently co-transfected COS-7 cells with plasmids containing cDNAs coding for four TPMT allozymes resulting from most common TPMT alleles (TPMT*1, *2, *3A, *3C) and a *lacZ* bearing reporter plasmid. We used monkey kidney COS-7 cells since they have been previously employed to study the heterologous expression of recombinant hTPMT allozymes [39] and since high transfection efficacy (>99%) is achieved using the approach described in Supplementary Methods. After transfection, cells were grown in Met+ or Met− media to ensure differential intracellular SAM concentration. Immunoblotting analysis was used to quantify TPMT protein expression and revealed that the recombinant allozymes (TPMT-R) migrated more slowly in polyacrylamide gels compared to the endogenous monkey TPMT (TPMT-E) (Fig. 5A). Under conditions of Met restriction, TPMT-R protein levels were significantly decreased in the case of TPMT*1 and TPMT*3C allozymes, with a more pronounced effect observed in the case of TPMT*3C (Fig. 5B).

Since steady state TPMT*3A and *2 protein levels were very low it is difficult to elaborate on further destabilization of the protein in Met− cells. Due to SAM depletion, TPMT-E protein levels were lower in all Met− compared to Met+ cell lysates. We also observed that in TPMT*2 and 3A lysates TPMT activity values were comparable to those in pcDNA3.1 mock transfected cells (Fig. 5C), indicating that low TPMT*2 and *3A recombinant allozyme levels did not contribute to the overall TPMT activity. In these lysates, decreased TPMT activity in Met− cells may be thus attributed solely to decreased stability of endogenous TPMT.

In the case of TPMT*1 and *3C both TPMT-R and TPMT-E contributed to the catalytic activity of COS-7 cell lysates. We observed a prominent effect of Met restriction on recombinant TPMT*1 and *3C proteins and TPMT activity indicating that these allozyme variants are susceptible to SAM-mediated stabilization at the cellular level.

3.6. Correlation between SAM concentration and TPMT activity in wild-type individuals

We next attempted to translate our in vitro evidence on post-translational stabilization of TPMT allozymes by SAM to an in vivo situation in humans. A study of the effect of erythrocyte SAM level and TPMT genotypes (*1/*1 and *1/*3) on TPMT activity in healthy individuals ($n = 159$) was carried out. Further details of the study population are given in Supplementary Table S1.

Erythrocyte TPMT activity of wild-type (*1/*1) and heterozygous (*1/*3) individuals differed significantly ($P < 0.001$, t-test) and genotype-to-phenotype correlation was 91.8% (Supplementary Fig. S5). Our initial statistical analysis showed that a significant positive correlation between log-transformed SAM concentration and TPMT activity data is observed in wild-type individuals (Pearson $R = 0.186$; $P = 0.046$) but not in heterozygotes (Supplementary Table S2). To examine the combined effect of TPMT genotype and SAM levels on TPMT activity, we used stepwise multiple linear regression modeling. As expected, a highly significant correlation between genotype and log-transformed TPMT activity ($\beta = 0.739$; $P < 0.001$) was observed. However, the overall goodness of fit was improved ($\beta = 0.749$) if log-transformed SAM concentration was included in the model (P-value for log SAM as an individual covariate was 0.026). Although the effect of SAM on TPMT activity was modest these results support the notion that SAM stabilizes TPMT and fine-tunes its activity (Fig. 6A).

To gain more insight into the impact of SAM on TPMT genotype and phenotype subgroups, subjects were divided into 4 sub-groups

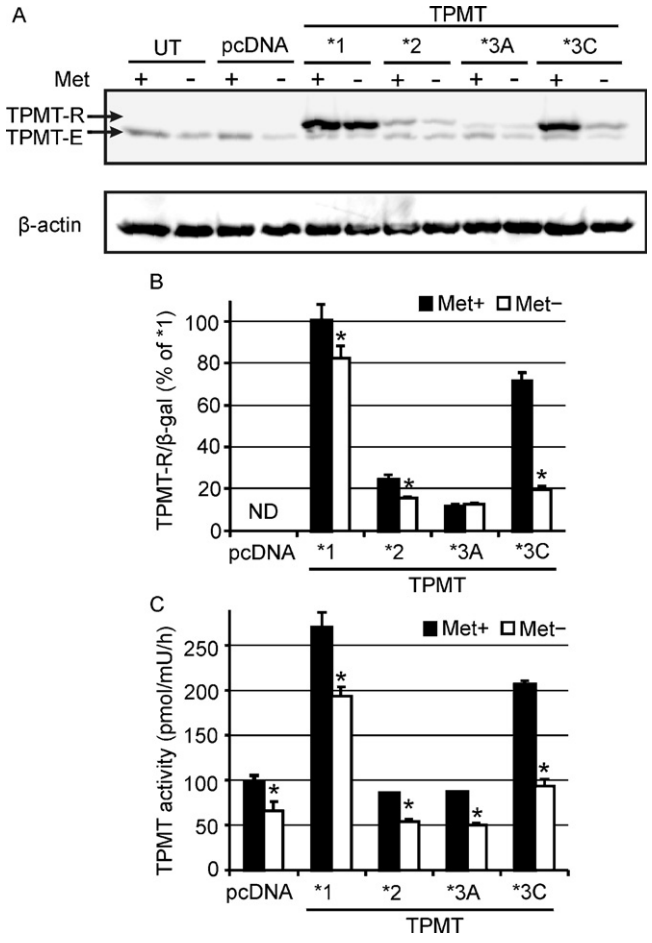

Fig. 5. Effect of Met availability on protein levels and activity of recombinant TPMT allozymes. COS-7 cells were co-transfected with reporter pcDNA3.1/Hygro/lacZ plasmid and pcDNA3.1 vectors containing cDNAs coding for four TPMT allozymes corresponding to the most common alleles (TPMT*1, *2, *3A, *3C). Untransfected (UT) and mock control (pcDNA) was included in the experiments. After transfection, cells were grown in Met+ DMEM overnight, followed by incubation in Met+ or Met− DMEM for 24 h and harvested. (A) Representative immunoblot of total lysates of cells expressing variant TPMT allozymes. TPMT-R, recombinant TPMT allozyme; TPMT-E, endogenous monkey kidney TPMT. (B) TPMT band intensity determined in (A) was normalized to β-gal activity and expressed as percentage of TPMT*1 Met+ band intensity. (*$P < 0.02$ vs. Met+ cells). (C) Total TPMT activity was determined in cell lysates and normalized to β-gal activity (*$P < 0.05$ vs. Met+ cells). ND, not determined.

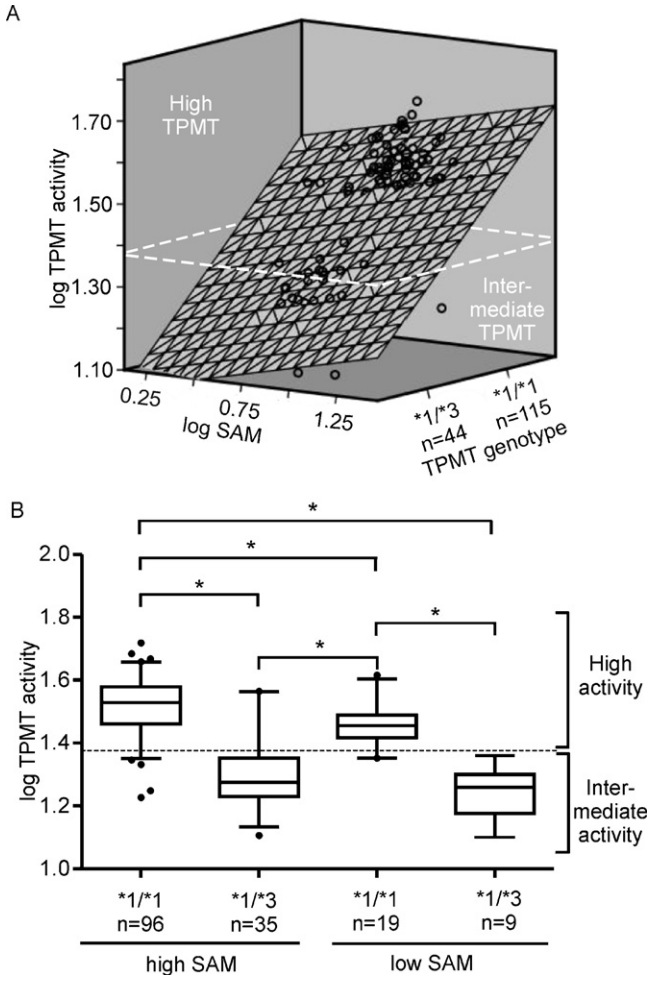

Fig. 6. Impact of erythrocyte SAM levels and TPMT genotype on TPMT activity in healthy individuals. (A) Multiple linear regression modeling of log-transformed TPMT activity, based on TPMT genotype and log-transformed SAM data ($n = 159$). Data points are depicted by empty circles, and black grid denotes the regression plane. The white dashed line denotes the ROC-defined cut-off (log TPMT = 1.38) between high and intermediate TPMT activity. (B) Subjects ($n = 159$) were divided into 4 subgroups based on SAM levels (high or low; defined by entropy-MDL) and TPMT genotype (*1/*1 or *1/*3). The dashed line denotes the ROC-defined cut-off between high and intermediate TPMT activity. Boxes and whiskers include data between 25th and 75th percentile, and between 5th and 95th percentile, respectively. Outliers are depicted as dots and median values as solid lines. Means of log-transformed TPMT activity were compared between groups by one-way ANOVA (*$P < 0.01$). *n*, number of subjects.

based on categorization of SAM levels (high or low) and genotype (*1/*1 or *1/*3). Since the frequency distribution of logSAM concentration was unimodal (not shown), the cut-off (log-SAM = 0.739) between low and high SAM was determined by the entropy-MDL method. The wild-type genotype and high SAM level group exhibited significantly higher TPMT activity than wild-type individuals with low SAM levels (Fig. 6B). Although heterozygous individuals with high erythrocyte SAM levels had higher mean TPMT activity than heterozygotes with low SAM levels, the difference was not significant.

Next, we determined if SAM measurements may enable the classification of subjects into additional TPMT phenotype subgroups, which were discretized as described in Supplementary Methods. Logistic regression modeling revealed that if wild-type individuals ($n = 115$) are divided into high-low (H^L) and high-high (H^H) subgroups, individuals with high SAM are more likely to have H^H than H^L activity (odds ratio (OR) = 26.31; 95% CI = 3.37–205.27; $P = 0.002$) (Supplementary Table S3). While no significant effect of SAM status was observed in individuals with TPMT*1/*3 genotype ($n = 44$), inclusion of all individuals independent of genotype ($n = 159$) displayed a similar trend than in the high activity group (OR = 2.12; 95% CI = 0.92–4.88; $P = 0.077$). Frequency distribution of high TPMT activity revealed that most individuals in the H^L group were also classed to have low erythrocyte SAM concentrations and vice versa (Supplementary Fig. S6).

4. Discussion

The aim of this study was to identify factors other than TPMT genotype, which influence TPMT activity and contribute to the efficacy and safety of thiopurine therapy. We addressed the question whether physiologic SAM levels may effectively modulate TPMT activity and evaluated the factors influencing SAM biosynthesis as potential regulators of TPMT activity.

Despite the fundamental role of TPMT in thiopurine treatment, little has been known about its intracellular regulation. SAM has been described as a modulator of a number of important genes via

transcriptional, post-transcriptional and post-translational mechanisms. Most notably, SAM post-translationally stabilizes catechol O-methyltransferase (COMT), a polymorphic enzyme involved in catabolism of catechol estrogens and catecholamines [40], as well as cystathionine β-synthase (CBS), the rate-limiting enzyme in the trans-sulfuration pathway [38].

Here we have uncovered the SAM-mediated post-translational stabilization of TPMT in live cells, and provided evidence that erythrocyte SAM levels impact basal levels of TPMT activity in healthy wild-type individuals. These findings may facilitate the identification of novel biomakers for improved prediction of thiopurine therapy outcomes.

SAM, a co-substrate in TPMT-catalyzed reactions, was shown in in vitro experiments under the conditions of high Met to prevent degradation of TPMT allozymes in cell lysates [27]. The tridimensional structure of the *Pseudomonas syringae* wild-type TPMT orthologue is stabilized by sinefungin, a SAM analogue [26]. We have addressed the issue of acute regulation of TPMT by SAM and factors, which participate or mediate its biosynthesis, as well as the metabolites involved in salvage pathways and transmethylation under physiological conditions. This was tested by culturing the cells in media lacking Met, and by subsequent addition of Met and its metabolites (SAM, MTA, FA, Hcy) to Met-depleted cells, as well as by using specific enzyme inhibitors. High intracellular SAM levels correlated positively with TPMT activity and protein levels, as SAM was found to maintain TPMT levels post-translationally by stabilizing it against proteasome degradation (Figs. 3 and S3). We also demonstrated that the increase in TPMT activity was a specific response to the addition of Met or SAM by incubation of Met− cells in PBS containing Met or SAM. In addition, inhibition of protein synthesis did not prevent the increase of TPMT activity after reintroduction of Met to Met− cells. These experiments indicated that fluctuations in TPMT activity are not due to an indirect response to Met restriction or supplementation (e.g. global increase in protein degradation or synthesis rates) but due to the interaction of Met or its metabolites with TPMT.

To identify the molecule responsible for TPMT stabilization, enzyme inhibitors of pathway enzymes were used to achieve differential levels of intracellular metabolites. Similar approaches in studying regulation of enzymes involved in Met metabolism in HepG2 cells have been described for MAT and CBS [37,38], and the concentrations of inhibitors and other substances used in our study were lower or identical (Fig. 4). We found that high intracellular levels of SAM but not Met or MTA are required to restore TPMT activity levels in Met− cells, and that the metabolites downstream of SAM do not modulate TPMT activity in HepG2 cells.

Apart from the wild-type TPMT*1 protein, we also demonstrated that intracellular SAM levels even more dramatically stabilize the TPMT*3C allozyme expressed in COS-7 cells, and this was reflected in enzyme activity measurements in cell lysates. We conclude that SAM stabilization has the greatest effect on those variant TPMT allozymes where the amino-acid substitutions have a relatively modest effect on the tridimensional structure of the enzyme, e.g. TPMT*3C [23], whereas the rapid degradation of TPMT*3A allozyme cannot be prevented by high intracellular SAM levels. However, in a clinical situation, assuming the supply of Met is sufficient, the presence of TPMT*3C allele causes only a relatively minor decrease in TPMT activity compared to the wild-type TPMT*1 allele, as previously reported [27].

The indirect effect on TPMT stabilization by the folate pathway, which supplies the necessary substrates for SAM biosynthesis via Hcy remethylation was also evaluated. Our results indicated that SAM concentration and TPMT stability may be significantly influenced by the folate pathway (Fig. 3). This indicates the relevance of folate supplementation (e.g. leucoverin) in patients with high risk of toxic events during maintenance ALL treatment, and may explain our earlier observation that patients with both heterozygous TPMT genotype and low-activity mutations in MTHFR (Fig. 1) had a significantly higher risk of hematotoxicity than patients with mutant TPMT genotype only [16].

Due to the fact that therapy optimization is based on TPMT genotyping or activity measurements, identification of additional biomarkers which markedly influence TPMT activity are extremely important to improve the genotype-to-phenotype correlation and predictive value of pharmacogenetic testing. We have demonstrated a great impact of SAM on TPMT activity in healthy individuals (Fig. 6). Hence, a subgroup of wild-type TPMT individuals, which had low erythrocyte SAM levels, exhibited significantly lower TPMT activity than a subgroup of individuals with high SAM levels. These findings are most relevant for high thiopurine methylators who are potential non-responders to thiopurine therapy. However, we observed no significant difference in TPMT activity between TPMT*1/*3 individuals with high or low SAM levels. Since TPMT*3A was the most frequent allele in our study population, these results may indicate that the susceptibility of TPMT*3A allozyme to proteasome degradation is too high to be prevented by SAM [23]. Further large-scale studies are needed to address the effect of SAM on TPMT activity in TPMT*1/*3C heterozygotes.

In summary, our results show that measurement of erythrocyte SAM level, in addition to TPMT genotyping, could serve as an additional predictor of TPMT activity in wild-type patients, and suggest that post-translational stabilization of TPMT by SAM has substantial clinical relevance. As an effective acute regulator of TPMT protein and activity levels, SAM may also be a promising agent in prevention of excess toxicity during thiopurine treatment, which remains to be determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2012.01.010.

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