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S-adenosylmethionine regulates thiopurine methyltransferase activity and decreases 6-mercaptopurine cytotoxicity in MOLT lymphoblasts

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ABSTRACT

Six-mercaptopurine (6-MP) is a pro-drug widely used in treatment of various diseases, including acute lymphoblastic leukaemia (ALL). Side-effects of thiopurine therapy have been correlated with thiopurine methyltransferase (TPMT) activity.

We propose a novel TPMT-mediated mechanism of S-adenosylmethionine (SAM)-specific effects on 6-mercaptopurine (6-MP) induced cytotoxicity in a model cell line for acute lymphoblastic leukemia (MOLT). Our results show that exogenous SAM (10–50 μM) rescues cells from the toxic effects of 6-MP (5 μM) by delaying the onset of apoptosis. We prove that the extent of methylthioinosine monophosphate (MeTIMP) induced inhibition of *de novo* purine synthesis (DNPS) determines the concentrations of intracellular ATP, and consequently SAM, which acts as a positive modulator of TPMT activity. This leads to a greater conversion of 6-MP to inactive 6-methylmercaptopurine, and thus lower availability of thioinosine monophosphate for the biotransformation to cytotoxic thioguanine nucleotides (TGNs) and MeTIMP. We further show that the addition of exogenous SAM to 6-MP treated cells maintains intracellular SAM levels, TPMT activity and protein levels, all of which are diminished in cells incubated with 6-MP. Since TPMT mRNA levels remained unaltered, the effect of SAM appears to be restricted to protein stabilisation rather than an increase of TPMT expression. We thus propose that SAM reverses the extent of 6-MP cytotoxicity, by acting as a TPMT-stabilizing factor.

This study provides new insights into the pharmacogenetics of thiopurine drugs. Identification of SAM as critical modulator of TPMT activity and consequently thiopurine toxicity may set novel grounds for the rationalization of thiopurine therapy.

1. Introduction

Thiopurines (e.g. 6-mercaptopurine, 6-MP; 6-thioguanine, 6-TG) are pro-drugs widely used in treatment of various diseases, including acute lymphoblastic leukaemia (ALL) [1]. The cytotoxic effect of 6-MP depends on two distinct metabolic pathways (Fig. 1) [2]. The conversion of 6-MP by hypoxanthine phosphoribosyltransferase (HPRT) yields thioinosine monophosphate (TIMP), which can either be converted to active thioguanine nucleotides (TGNs), or methylated by thiopurine methyltransferase (TPMT; EC 2.1.1.67) to methylthioinosine monophosphate (MeTIMP) [3]. Incorporation of TGNs into DNA and RNA results in S phase arrest [4] and programmed cell death [5], triggered via the mismatch repair pathway [6]. On the other hand, MeTIMP is a potent inhibitor of *de novo* purine synthesis (DNPS), causing depletion of purine nucleotides, which results in cell growth arrest and cytotoxicity [7]. Nevertheless, the incorporation of TGNs is considered to be the main mode of action of 6-MP [8].

TPMT also catalyzes the direct methylation of 6-MP to produce the inactive 6-methylmercaptopurine (6-MMP) leading to lower toxic potential of the drug and consequently to higher leukemic cell survival (Fig. 1) [9]. Due to the presence of genetic polymorphisms, heterozygous and homozygous individuals for mutant alleles display intermediate and low TPMT activity, respectively [10,11]. ALL patients with decreased TPMT activity require dosage adjustment protocols, since treatment with standard doses of 6-MP results in high cytotoxic TGN levels, causing severe life-threatening toxicity [12,13]. Molecular studies have shown that non-synonymous amino-acid substitutions in mutant TPMT isoforms cause the disruption of intra-molecular van der Waals contacts [14]. Consequently, such variant proteins are readily degradable via proteasome-mediated proteolysis [15], a process which can be partially prevented by S-adenosylmethionine (SAM), the methyl donor in the S-methylation reactions catalyzed by TPMT [16]. The binding of SAM has been shown to stabilise the 3D structure of the enzyme [17].

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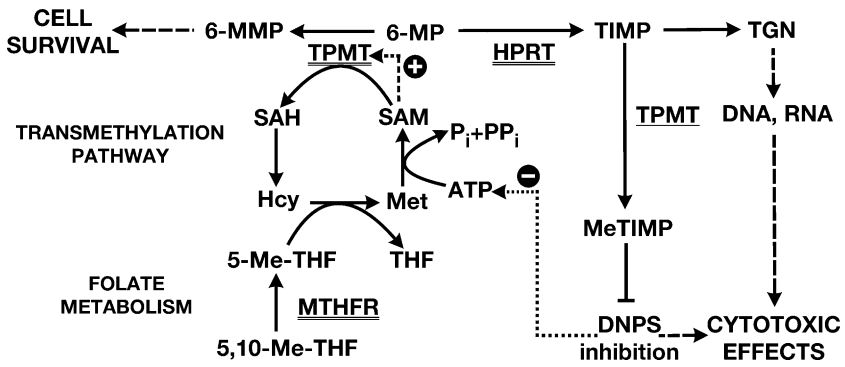

Fig. 1. The role of SAM in the metabolic pathway of 6-MP. In TPMT-catalyzed reactions, SAM acts as a methyl donor, but can also stabilize the TPMT 3D structure (indicated by plus, encircled). Inhibition of DNPS by MeTIMP results in depletion of ATP (minus, encircled), limiting the recycling of SAM from methionine. SAH, S-adenosylhomocysteine; Hcy, homocysteine; Met, methionine; 5-Me-THF, 5-methyltetrahydrofolate; THF, tetrahydrofolate; 5,10-Me-THF, 5,10-methylenetetrahydrofolate; MTHFR, 5,10-methylenetetrahydrofolate reductase.

With respect to the thiopurine toxicity, decreased SAM recycling appears to be a consequence of MeTIMP inhibition of DNPS [18], since the ATP-dependent synthesis of SAM from methionine is limited [19]. In addition, the presence of low-activity mutations in 5,10-methylenetetrahydrofolate reductase (MTHFR), the enzyme that catalyzes the formation of 5-methyltetrahydrofolate, a rate-determining step for the conversion of methionine to homocysteine (Fig. 1) has been found to correlate with decreased TPMT activity [20] and toxic side-effects of 6-MP in patients with ALL [21]. It was postulated that low MTHFR activity results in limited SAM synthesis and consequently lower TPMT stability.

Since low TPMT activity is strongly correlated with unfavourable side-effects during thiopurine treatment, availability of endogenous SAM could contribute to the extent of 6-MP induced cytotoxic effects. To test this hypothesis, we used a human ALL cell line MOLT, and found that SAM-specific effects include the regulation of thiopurine metabolism by acting as a TPMT-stabilizing factor, resulting in decreased amounts of cytotoxic 6-MP metabolites, and diminished cytotoxic effects in these cells.

2. Materials and methods

2.1. Chemicals

Allopurinol, 6-MP, 6-MMP, MMPR, 6-TG, SAM, N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), dithiothreitol (DTT), DMSO, ATP and propidium iodide (PI) were from Sigma Chemical Co. (St. Louis, MO, USA). For cell culture applications, stock solutions of 6-MP (225 mM in DMSO) and SAM (100 mM in PBS) were filtered (0.2 μm) and diluted in cell culture medium to the appropriate concentration, prior to the addition to cells. In the case of vehicle-treated controls, cell culture media containing the same amount of DMSO or PBS were added. Perchloric acid, ethanol, HPLC-grade acetonitrile and methanol were from Sigma.

2.2. Cell culture

Human T-lymphoblastic cell line MOLT was purchased from American Type Culture Collection (LGC Promochem, Wesel, Germany) and grown in RPMI 1640 medium (Sigma), supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and 50 μM 2-mercaptoethanol (all from Sigma). Cells were incubated in a sterile environment at 37 °C with a humidified atmosphere of 5% CO_2, and maintained in the density range of 0.4–2.0 × 10^6/ml. In all experiments, the concentration of cells at the beginning of incubation with 6-MP, SAM, or vehicles ($t = 0$) was 0.5 × 10^6/ml.

2.3. Trypan blue exclusion assay

Cells (5.0 × 10^5/ml) were incubated in culture media containing 5 μM 6-MP, 25 μM SAM, or vehicles for different time periods. Afterwards, the cells were suspended in 0.2% Trypan Blue solution (Sigma), stained for 5 min, and counted on a haemocytometer as described previously [22]. Viability (%) was determined by dividing the number of viable cells (white) by the total cell count (white and blue).

2.4. MTS assay

Metabolic activity of cells was determined by the CellTiter Aqueous One Proliferation Assay™ (Promega, Madison, WI, USA) as described previously [23]. Briefly, cells (1.5 × 10^4/well) were seeded into 96-well plates and incubated in culture media containing different concentrations of 6-MP, SAM, or vehicles for 24 or 48 h. Afterwards, the MTS reagent (1:10) was added and the plates were incubated for another 3 h at 37 °C. Absorbance (492 nm) readings were carried out by an automated plate reader (Tecan Safire2, Austria). Assays performed without cells served as background controls.

2.5. Detection of apoptotic cells

The Annexin V-FITC Apoptosis Detection Kit™ (Sigma) was used to detect apoptotic cells, according to the manufacturer's instructions. Flow-cytometric analysis was carried out immediately after staining on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany), utilizing the fluorescence detectors FL1 (530 nm) and FL3 (679 nm) for the detection of Annexin V-FITC and PI, respectively. Percentages of Annexin V^+/PI^- (early apoptotic) and Annexin V^+/PI^+ (dead) cells were determined by CellQuest Pro software (BD Biosciences).

2.6. DEVDase activity

Caspase-3 activity was measured in total cell lysates using a labelled Ac-DEVD.AFC peptide substrate (Sigma) as described previously [24]. Incubation of cells with 100 μM TPCK was used as a positive control of caspase-3 induction. Data were expressed as increase in fluorescence intensity as a function of time (dF/dt).

2.7. Cell cycle analysis

The DNA content of cells was determined by the flow cytometric analysis as previously described [22], with the exception that cells (2.0 × 10^6) were fixed in 80% ethanol at −20 °C overnight.

2.8. Fluorescence microscopy

The DNA intercalating Hoechst 33342 dye (Sigma) was used for live-cell fluorescence imaging of nuclei. Cells (1.2×10^6) were washed in PBS and stained with Hoechst (5.0 μg/ml) for 20 min at room temperature. Afterwards, the cells were washed twice, resuspended in PBS (1.5×10^6/ml), placed into a cytospin chamber (Cytospin2, StatSpin Inc., Norwood, MA, USA), and centrifuged (1100 rpm, 10 min). Fluorescence imaging was carried out immediately on an Olympus IX 81 inverted microscope (Tokyo, Japan) with the 360 nm/420 nm excitation/emission filter to visualize the Hoechst-stained nuclei. Images were taken at a total magnification of 960x and processed by the Cell^R software (Olympus).

2.9. Cytosolic TGN levels

Intracellular TGNs were measured by the modified reversed-phase HPLC method described previously [25,26]. Cells (3.0×10^6) were washed twice, resuspended in PBS and frozen at −80 °C. The cells were lysed by multiple freezing and thawing cycles, followed by centrifugation (14,000 × *g*, 5 min, 4 °C). After the addition of 70% $HClO_4$ (10 μl) and 0.65 M DTT (10 μl), the samples were incubated on ice (10 min) and centrifuged. The supernatants were hydrolyzed by heating (100 °C, 75 min) to release 6-TG. Afterwards, they were immediately placed on ice and re-centrifuged to remove any remaining debris. Aliquots (40 μl) were separated on a Zorbax ODS column (25 cm × 0.46 cm, 5 μm; Agilent Technologies, Santa Clara, CA, USA) utilizing an automated Agilent Technologies HPLC 1100 system by isocratic elution (10 mM potassium phosphate buffer, pH 2.2, 4% acetonitrile) at a flow rate of 1.0 ml/min. Integration of peaks was carried out by the Chemstation for LC 3D Systems software (Agilent). The concentration of TGNs is reported as the amount (pmol) of 6-TG present after hydrolysis per 10^6 viable cells.

2.10. Cytosolic MeTIMP levels

Methylated metabolites of 6-MP were detected as MMPR after the digestion of cell lysate with alkaline phosphatase (AP), according to the previously described reversed-phase HPLC method [27], with minor modifications. Cells were lysed as for TGN determinations. The resulting supernatants (85 μl), with the addition (10 μl) of 0.5 M Tris buffer (pH 7.8), were treated with calf intestine AP (10 U, Calbiochem, Gibbstown, NJ, USA) for 2 h at 37 °C. 1.8 M $HClO_4$ (10 μl) was then added and the samples were incubated on ice (10 min). After centrifugation (14,000 × *g*, 5 min, 4 °C), the supernatants (aliquots of 70 μl) were used for HPLC analysis. MMPR was eluted isocratically in 180 mM potassium phosphate buffer, pH 6.85, supplemented with 15% acetonitrile, at a flow rate of 1.0 ml/min, and was detected at 290 nm. HPLC instrumentation was the same as above and the concentration of MeTIMP was reported as the amount (pmol) of MMPR present per 10^6 viable cells.

2.11. Intracellular ATP levels

ATP concentrations were determined by the CellTiter Glo Luminescent Cell Viability Assay™ (Promega) according to the manufacturer's instruction. After incubation with the chemicals, viable cells were plated into 96-well opaque microtiter plates (NalgeNunc, Roskilde, Denmark) (1.0×10^4 cells per well) and incubated with the CellTiter Glo reagent (1:1) for 2 min on an orbital shaker to induce cell lysis and facilitate the ATP-dependent luciferase reaction, resulting in the production of oxyluciferin. The plates were then equilibrated at room temperature for 10 min, and luminescence was recorded on an automated plate reader (Tecan Safire2, Zürich, Switzerland). Standards solutions of ATP were prepared in culture media on the same plate and processed as described above. The concentration of ATP was reported as the amount (pmol) of ATP per 10^4 viable cells.

2.12. Intracellular SAM levels

Cells (8.0×10^6) were washed twice in PBS and an aliquot was stored at −80 °C for determination of protein concentration with the BioRad Protein Assay Kit (Hercules, CA, USA) according to the manufacturer's instructions. The remaining pellets were lysed in 0.5 M $HClO_4$ (100 μl) for 20 min on ice. After centrifugation (14000 × *g*, 2 × 10 min, 4 °C), SAM was measured in the supernatants by the reversed-phase HPLC method essentially as described previously [28]. The concentration of SAM was reported as the amount (nmol) of SAM per mg of protein.

2.13. TPMT activity

TPMT activity was measured by the reversed-phase HPLC method essentially as described previously [10,29]. To report the TPMT activity as the amount (pmol) of 6-MMP formed per mg of protein per hour, an aliquot of the reaction mixture was used to determine protein concentration (see above).

2.14. TPMT protein level

TPMT protein level was determined by flow cytometric analysis of immunofluorescence resulting from cells stained for TPMT. Cells (2.0×10^6) were washed twice in PBS and fixed in 0.25% ice-cold paraformaldehyde (Sigma) for 1 h. The cells were then pelleted and incubated in permeabilization buffer (0.2% Tween in PBS) for 15 min at 37 °C. After incubating briefly in 3% BSA (Sigma), monoclonal anti-TPMT antibody (Abnova, Taipei, Taiwan) diluted in PBS (20 μg/ml) was added. Incubation for 1 h on ice was followed by washing the cells in permeabilization buffer, and the pellet was again incubated briefly in 3% BSA, followed by the addition of fluorescein isothiocyanate (FITC)-conjugated secondary goat anti-mouse IgG antibody (Molecular Probes, Invitrogen) diluted in PBS (100 μg/ml). After incubation for 30 min on ice, the cells were washed twice, resuspended in PBS and analyzed on a FACSCalibur flow cytometer (BD Biosciences), detecting the FITC fluorescence intensity at 530 nm (FL1). For background fluorescence gating, cells were stained with the secondary antibody alone. TPMT protein expression was reported as the percentage of TPMT-stained cells in the range of <5% false positive cells stained with secondary antibody alone. Data analysis was carried out with CellQuest Pro software (BD Biosciences).

2.15. TPMT mRNA level

Total RNA was extracted from 4.0×10^6 cells using Trizol Reagent (Invitrogen) according to the manufacturer's instruction. RNA concentration was determined on an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and the integrity was checked by electrophoresis on an Agilent Technologies 2100 bioanalyzer. Reverse transcription (RT) and quantitative PCR were carried out as described previously [30], by using Omniscript RT kit (Qiagen, Germantown, MD, USA), and a TPMT-specific Taqman Gene Expression assay (AssayID: Hs00909011_m1, Applied Biosystems, Foster City, CA, USA), relative to the expression of human cyclophilin A. Data were analyzed according to the modified comparative C_t method of Pfaffl [31].

2.16. Statistical analysis

Results were expressed as means ± S.D. from three independent experiments. Student's *t*-test was used for between-group comparisons, and the Spearman rank test (*r*) was used for correlations between parameters. All statistical analyses were carried out using Microsoft Office Excel 2003 (Microsoft Corporation) and SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). A 2-sided *P* value of 0.05 or less was considered statistically significant.

3. Results

3.1. SAM reverses the cytostatic and cytotoxic effects of 6-MP on MOLT cells

The cytotoxicity induced by 6-MP was reversed by exogenous SAM in a concentration and time dependent manner in MOLT cells. This cell line serves as a model for 6-MP sensitivity [4] and exhibits constitutive TPMT activity [29]. The analysis of genomic DNA of these cells revealed the absence of the three most prevalent low-activity mutant TPMT alleles (*2, *3A and *3C; data not shown).

Cell viability and proliferation experiments showed that MOLT cells were sensitive to the presence of 6-MP in the range of 1–20 μM. Proliferation was reduced to approximately 40% of the untreated control after 48-h incubation with 5 μM 6-MP (Fig. 2A and C) ($P < 0.01$). This concentration was used for all further experiments, since its effects were in accord with other published data [4]. Approximately 80% of metabolic activity remained when cells were co-treated with 5 μM 6-MP and 25 μM SAM (Fig. 2B and C), indicating a reversal of 6-MP induced growth inhibition. Incubation with only 25 μM SAM had no effect on cell proliferation. This concentration was used for all further experiments.

In addition, we assessed the viability of cells to determine whether the cause of SAM-induced restoration of cell proliferation was also the cause of reduction in cytotoxic effects of 6-MP (Fig. 2D). Approximately 25% reduction in viability was observed when cells were treated with 6-MP for 48 h, while co-treatment with 6-MP and SAM resulted in a protective effect of SAM on 6-MP induced cell death; a 6% reduction in viability was observed when compared to the untreated cells ($P < 0.01$). Therefore, the extent of both cytostatic and cytotoxic effects of 6-MP depends on the presence of exogenous SAM.

3.2. SAM delays and limits the onset of 6-MP induced programmed cell death

SAM displayed protective effects on 6-MP induced cytotoxicity on the molecular and cellular levels, as confirmed by differences in caspase-3 induction, annexin V staining, cell cycle distribution, and DNA fragmentation (Fig. 3). In 6-MP treated cells, caspase-3 activity peaked after 24 h of incubation, while co-treatment with 6-MP and SAM resulted in maximum caspase-3 activity after 48 h (Fig. 3A). Caspase-3 activity in the co-treated sample after 24 h was approximately 60% of that observed in cells treated with 6-MP ($P < 0.01$).

To investigate the effect of SAM on the progression of cells to cell death, live cells were double-stained with annexin V and propidium iodide (PI). Flow cytometric analysis revealed that 30% of 6-MP treated cells were apoptotic after 48 h of incubation, consisting of equal percentages of dead (annexin V^+/PI^+) and pro-apoptotic (annexin V^+/PI^-) cells (Fig. 3B). This effect was partially reversed in the case of simultaneous treatment with SAM, resulting in 20% of the cells being apoptotic ($P = 0.04$).

Cell cycle distribution after 48 h of incubation with 6-MP and/or SAM was determined by flow cytometric analysis of ethanol-fixed cells stained with PI (Fig. 3C). The histogram representing the cell cycle profile of cells treated with 6-MP reflects the features of apoptotic population, characterized by high proportion of cells in $subG_0/G_1$ phase (24%), increased fraction of cells in the S phase (38%), and a small population in G_2/M phase cells (8%), indicating a 6-MP-induced S phase arrest. However, co-treatment of cells with 6-MP and SAM had a less severe impact. The fraction of cells in the S phase was moderately increased compared to untreated cells (30% vs. 21%; $P < 0.02$), and additional accumulation of cells in G_2/M phase was observed (22% vs. 14%; $P < 0.01$). These results demonstrate that the presence of SAM enables the progression of cells through the S phase, a process strongly inhibited when cells are incubated with 6-MP alone.

Nuclear staining (Fig. 3D) revealed numerous cells with fragmented nuclei in 6-MP treated cultures, and only partially condensed but not fragmented nuclei in cultures co-treated with 6-MP and SAM.

These results suggest that SAM has no direct anti-apoptotic effects but rather delays the onset of downstream apoptotic signalling.

3.3. The concentration of cytotoxic 6-MP metabolites is lower in the presence of exogenous SAM

Cytosolic TGNs and MeTIMP are considered to be responsible for the cytotoxic effects of 6-MP, while the metabolic conversion to inactive 6-MMP diminishes these effects (Fig. 1) [6,7]. The addition of exogenous SAM influences drug metabolism dynamics and lowers the concentrations of TGNs and MeTIMP (Fig. 4).

Cytosolic TGNs and MeTIMP were determined in cell lysates by HPLC after different times of incubation (Fig. 4). The concentration of TGNs was significantly higher ($P < 0.01$) when cells were incubated with 6-MP alone than in the case of co-treatment with 6-MP and SAM after 8 and 24 h (Fig. 4A). After 48 h of incubation, the concentration of TGNs was also higher in cells treated with 6-MP alone than with both 6-MP and SAM, although the *P* value was slightly above the significance threshold (239.6 ± 53.3 vs. 171.5 ± 38.1 pmol/10^6 viable cells; $P = 0.07$). Similarly, MeTIMP concentrations were also significantly lower ($P < 0.05$) in the cells treated with both 6-MP and SAM than in cells treated with 6-MP alone, at all times studied (Fig. 4B). Therefore, the reversal of cytostatic and cytotoxic effects, as well as the slower onset of apoptosis in the presence of SAM, can be explained by the lower production of both TGNs and MeTIMP.

3.4. The concentration of MeTIMP determines the intracellular levels of ATP and SAM

To investigate the extent of MeTIMP induced inhibition of DNPS on re-synthesis of SAM, the intracellular levels of ATP and SAM were analyzed in cells either treated with 6-MP or co-treated with 6-MP and SAM. Higher concentrations of ATP and SAM were detected in cell lysates of co-treated compared to 6-MP treated cells.

A significant ($P < 0.01$) reduction in ATP concentration in 6-MP treated cells compared to untreated controls was determined after all times studied (Fig. 5A). This effect was partially reversed by the addition of SAM ($P < 0.05$). Our data show that the concentration of MeTIMP was inversely correlated with ATP levels in the lysates of 6-MP, as well as 6-MP and SAM treated cells (6-MP: $r = -0.99$; $P < 0.001$; 6-MP and SAM: $r = -0.89$; $P = 0.02$), while no correlation of ATP and TGN concentrations was observed (6-MP: $r = -0.49$; $P = 0.11$; 6-MP and SAM: $r = -0.52$; $P = 0.08$). Therefore, intracellular ATP levels are determined by the concentration of the DNPS inhibitor MeTIMP in both 6-MP treated samples, which shows that exogenous SAM acts upon the processes upstream rather than downstream of MeTIMP and ATP production.

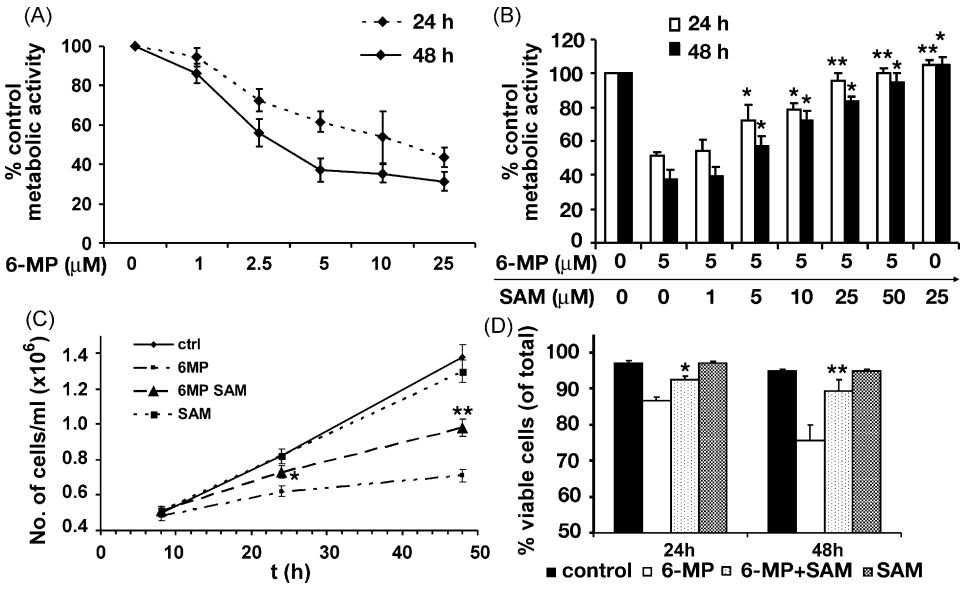

Fig. 2. SAM reverses the cytostatic and cytotoxic effects of 6-MP in MOLT cells. Cell proliferation and viability were assessed by MTS (A, B) and Trypan blue exclusion assays (C, D) following the incubation of cells with 6-MP and/or SAM for the indicated time periods. The asterisks indicate significant differences from the 6-MP treated sample (*t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$). (A) The decrease in cell proliferation by 6-MP was concentration- and time-dependent. (B) SAM (10-50 μM) reversed the decrease in cell proliferation caused by 6-MP (5 μM) in a dose-dependent manner. (C) The total cell count was higher after 24- and 48-h incubation with 6-MP (5 μM) and SAM (25 μM) together than with 6-MP (5 μM) alone, indicating a higher proliferation rate. (D) Cell viability was higher after co-treatment with 6-MP (5 μM) and SAM (25 μM) than with 6-MP (5 μM) alone.

Endogenous SAM has been shown to be depleted in MOLT cells incubated with 6-MP, since its endogenous synthesis from methionine requires ATP, which is depleted as a result of DNPS inhibition [19]. We observed a significant decrease in intracellular SAM levels ($P < 0.05$) when cells were treated with 6-MP, which is in accord with previously published data (Fig. 5A). However, in cells co-treated with 6-MP and SAM for 24 h, no depletion of intracellular SAM levels was observed ($P = 0.02$), indicating that

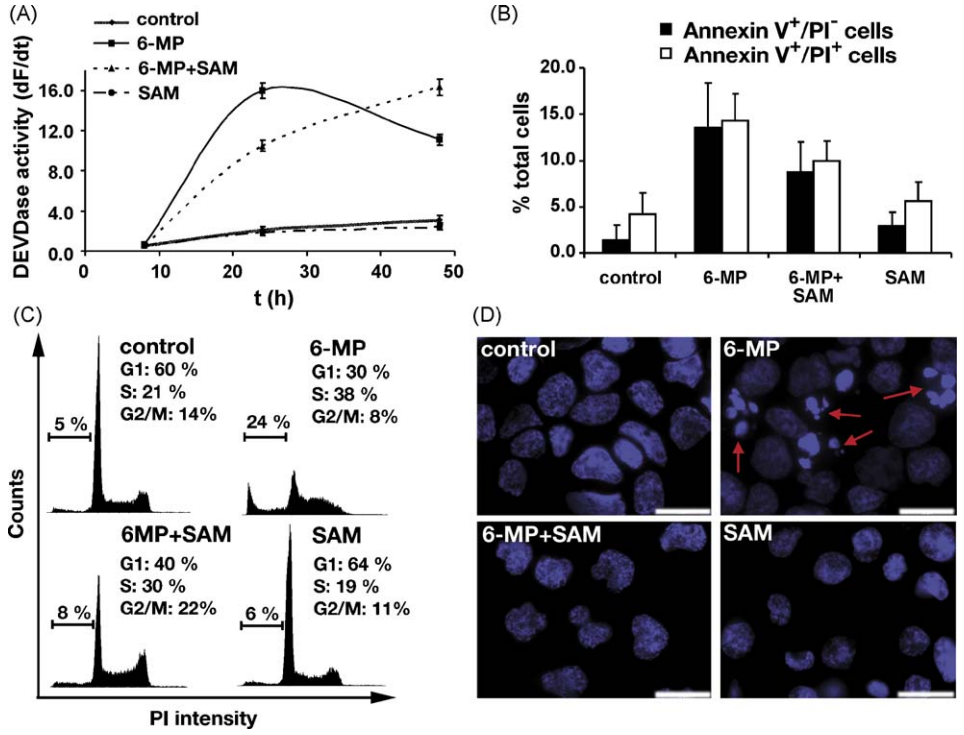

Fig. 3. SAM delays the onset of 6-MP induced programmed cell death in MOLT cells. Apoptotic features of cells were evaluated after incubation with 6-MP (5 μM) and/or SAM (25 μM) for the designated time periods. The asterisks indicate significant differences from the 6-MP treated sample (*t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$). (A) Caspase-3 activity was determined in whole cell lysates after the indicated time periods by recording continuous fluorescence intensity changes (excitation, 405 nm; emission, 535 nm) resulting from the cleaved Ac-DEVD.AFC substrate. (B) Live cells were stained with annexin V-FITC and PI after 48 h of incubation to determine the fractions of early apoptotic (annexin V^+/PI^-) and dead cells (annexin V^+/PI^+) by flow cytometric analysis. (C) Cell cycle analysis was performed after 48-h incubation in the presence or absence of 6-MP and/or SAM on ethanol-fixed cells stained with propidium iodide. (D) Representative images after 48 h of incubation of live cells stained with the nucleus-specific Hoechst 33342 dye. Evidence of nuclear fragmentation is indicated by red arrows. Magnification, 960×; scale bar, 20 μm.

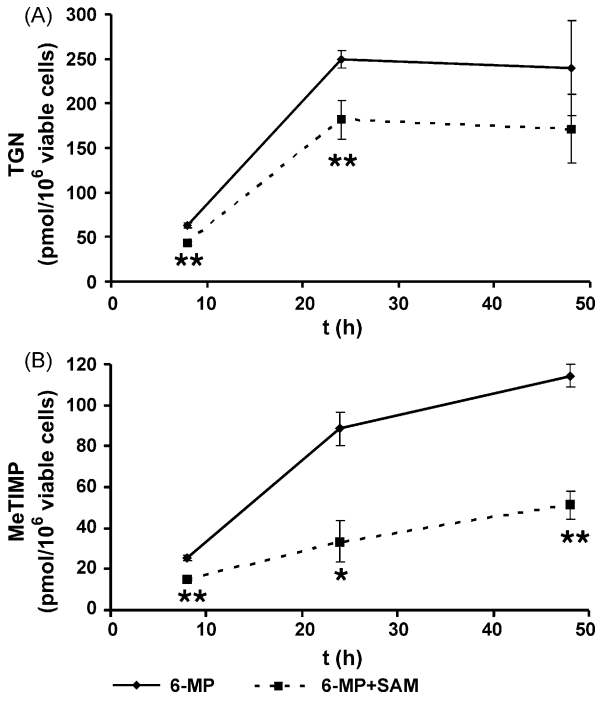

Fig. 4. The concentration of toxic 6-MP metabolites is lower in the presence of exogenous SAM. After the incubation of cells with 6-MP (5 μM), or 6-MP (5 μM) and SAM (25 μM), for the time periods indicated, the concentrations of cytosolic thioguanine nucleotides (TGNs) and methylthioinosine monophosphate (MeTIMP) were determined in cell extracts by HPLC. The asterisks indicate significant differences from the 6-MP treated sample (*t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$). (A) TGNs were measured as the amount of 6-thioguanine present in cell lysates after acid hydrolysis. The concentration of TGNs present in cells treated with 6-MP was higher than in the case of co-treatment with 6-MP and SAM [8 h: 63.0 ± 2.3 vs. 43.1 ± 2.2 ($P = 0.00002$); 24 h: 249.2 ± 10.0 vs. 181.6 ± 22.2 ($P = 0.001$); 48 h: 239.6 ± 53.3 vs. 171.5 ± 38.1 pmol/10^6 viable cells ($P = 0.07$)]. (B) MeTIMP was measured by HPLC as the amount of methylmercaptopurine riboside present after the digestion of the cell lysate with alkaline phosphatase. The concentration of MeTIMP present in cells co-treated with 6-MP and SAM was significantly lower than that with 6-MP alone at all times studied [8 h: 15.0 ± 0.5 vs. 25.4 ± 1.1 ($P = 0.007$); 24 h: 33.5 ± 10.4 vs. 88.6 ± 8.1 ($P = 0.03$); 48 h, 51.2 ± 7.2 vs. 114 ± 5.7 pmol/10^6 viable cells ($P = 0.006$)].

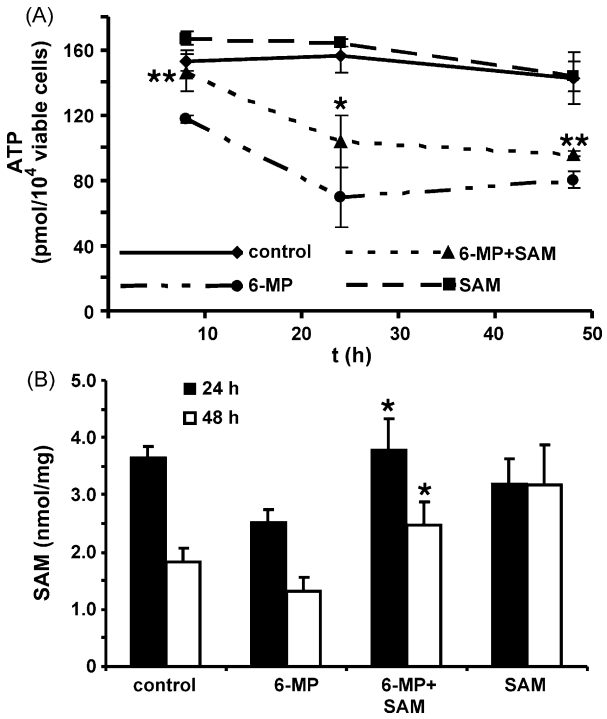

Fig. 5. Exogenous SAM maintains the intracellular levels of ATP and SAM. The asterisks indicate significant differences from the 6-MP treated sample (*t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$). (A) Intracellular ATP was determined by the ATP-coupled CellTiterGlo luminescence assay after the designated times of incubation in treated and control cells. Cells treated with both 6-MP (5 μM) and SAM (25 μM) displayed higher concentrations of ATP than cells treated with 6-MP (5 μM) alone at all times studied [8 h: 145.9 ± 11.5 vs. 116.8 ± 2.3 ($P = 0.003$); 24 h: 103.6 ± 15.9 vs. 69.7 ± 18.4 ($P = 0.03$); 48 h: 95.83 ± 1.7 vs. 80.1± 5.5 pmol/10^4 viable cells ($P = 0.001$)]. (B) The concentration of intracellular SAM was measured in cell extracts by a reversed-phase HPLC method. The concentration of SAM present in cells co-treated with 6-MP (5 μM) and SAM (25 μM) was significantly lower than that with 6-MP (5 μM) alone at all times studied [24 h: 3.80 ± 0.53 vs. 2.52 ± 0.22 ($P = 0.02$); 48 h: 2.47 ± 0.41 nmol/mg vs. 1.31 ± 0.26 nmol/mg ($P = 0.02$)].

SAM levels remain unchanged as a result of addition of exogenous SAM. After 48 h, endogenous SAM levels were lowered also in untreated cells, when compared to 24-h incubation, which may be attributed to the lower availability of SAM precursors (e.g. methionine, folic acid) in culture media.

3.5. SAM maintains the level of TPMT protein and activity

To address the molecular basis of the regulatory effects of SAM on 6-MP metabolism, TPMT enzyme activity, protein and mRNA levels were analysed after incubation with 6-MP and/or SAM (Fig. 6). Incubation of cells with 6-MP leads to a decreased TPMT activity and protein level, an effect which was reversed in the presence of SAM, indicating that SAM maintains TPMT activity levels.

For TPMT activity, the concentration of 6-MMP formed per unit time was determined by HPLC in samples of cell lysates incubated with the substrate 6-MP (2.25 mM; Fig. 6A). Lower TPMT activity ($P < 0.001$) was observed in 6-MP treated than in untreated cells. However, when cells were co-treated with 6-MP and SAM the level of TPMT activity was not significantly different from that in control and SAM-treated cells ($P > 0.05$). The concentration of intracellular SAM (Fig. 5B) correlated with TPMT activity ($r = 0.481$; $P = 0.02$), indicating that TPMT may be regulated by SAM through post-translational stabilisation.

Correspondingly, specific effects of 6-MP and/or SAM were also evident on the protein level, as determined by cytoplasmic staining of TPMT followed by flow cytometric analysis (Fig. 6B). A higher percentage of cells was found in the TPMT-positive range in cells co-treated with 6-MP and SAM for 48 h, than with 6-MP alone (59.0% vs. 51.1%). The TPMT protein level was thus less reduced in cells co-treated with 6-MP and SAM than in cells treated with 6-MP alone ($P < 0.01$). It should be noted that protein expression was analysed by flow cytometry since all immunoblotting experiments failed to give a satisfactory signal with low background staining, although several anti-TPMT antibodies from various commercial and custom-made sources were tested.

The decreased TPMT protein level was not due to lower TPMT mRNA expression, as determined by quantitative PCR (Fig. 6C). No significant changes in TPMT expression were observed as a result of treatment of cells with 6-MP or with 6-MP and SAM ($P > 0.05$). SAM thus appears to maintain the level of TPMT activity, most probably by post-translational stabilisation of the protein.

4. Discussion

The antiproliferative effects of cytotoxic metabolites of 6-MP have been extensively studied, both in vitro [19] and in vivo [12,32]. However, very few studies have addressed the impact of endogenous metabolites on drug metabolism [33]. TPMT is a highly polymorphic enzyme, which catalyzes the S-methylation and consequent deactivation of 6-MP, but may also contribute to cytotoxicity through the formation of MeTIMP, a potent DNPS inhibitor [3]. In the S-methylation reaction, SAM acts as a co-substrate by providing the methyl group, but also stabilises the

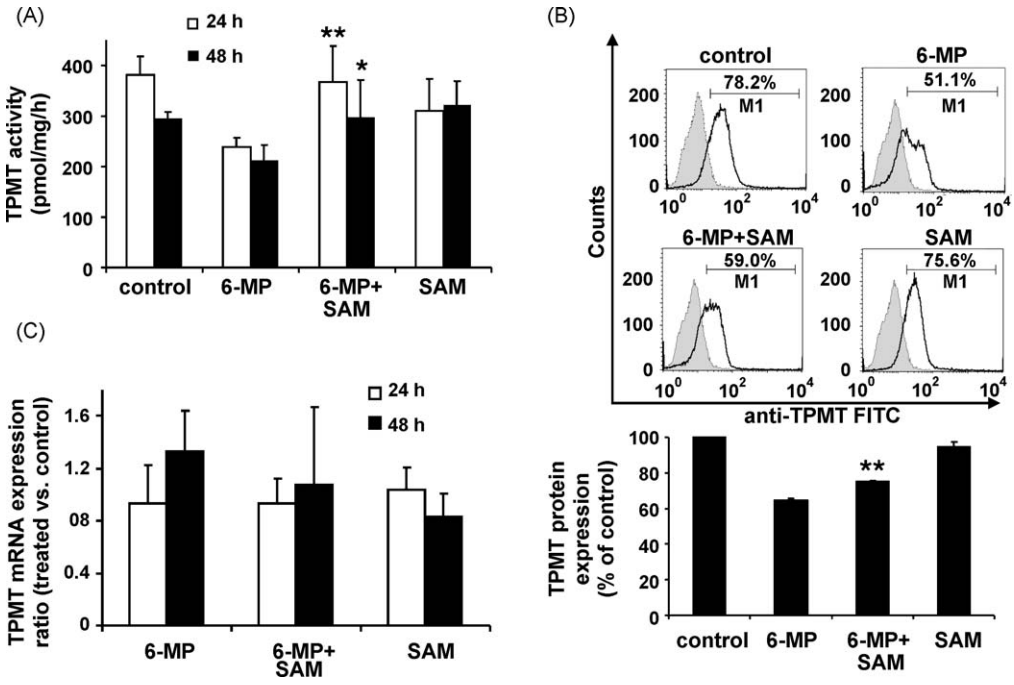

Fig. 6. SAM maintains the level of TPMT protein and activity. The asterisks indicate significant differences from the 6-MP treated sample (*t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$). (A) TPMT activity was followed by HPLC analysis in cells incubated for 24 or 48 h with 6-MP (5 μM), or with 6-MP (5 μM) and SAM (25 μM). Cell lysates were incubated with the substrate 6-MP (2.25 mM) and TPMT activity is reported as the amount of 6-MMP formed after the 2-h incubation. TPMT activity in the cells treated with 6-MP alone was found to be lower than in untreated control [24 h: 238.3 ± 18.1 vs. 381.7 ± 35.0 ($P < 0.001$); 48 h: 212.0 ± 31.1 pmol/mg/h vs. 296.1 ± 11.1 pmol/mg/h ($P < 0.001$)], while the activity of cells treated with both 6-MP and SAM was unchanged [24 h: 365.5 ± 72.9 ($P > 0.05$); 48 h: 297.4 ± 73.6 pmol/mg/h ($P > 0.05$)]. (B) Representative histograms of flow cytometric analysis of TPMT protein levels after 48 h of incubation (top). TPMT was detected in permeabilized cells by a secondary FITC-conjugated anti-mouse IgG antibody bound to a monoclonal anti-TPMT antibody. TPMT expression was reported as the percentage of TPMT-stained cells (solid line, transparent peak) in the range (M1) of <5% false positive (control) cells stained with secondary antibody alone (dotted line, grey peak). TPMT protein expression is presented graphically as percent of expression in control cells from three independent experiments ± S.D. (bottom). (C) Total RNA was isolated from cells after 24 and 48 h of incubation, the mRNA was reverse transcribed and TPMT cDNA quantified by the Taqman gene expression assay. The expression ratio was calculated as the relative amount in the treated to control samples.

protein structure on binding into the active site of TPMT (Fig. 1) [17]. Since low TPMT activity is strongly correlated with unfavourable side-effects during thiopurine treatment [34], understanding of the role of SAM would enable prediction and modulation of 6-MP induced cytotoxic effects. In this study, human ALL cell line MOLT was used which serves as a relevant model to study the cytotoxic mechanisms and metabolism of thiopurines. We herein propose a novel TPMT-mediated mechanism of SAM-specific effects in the context of 6-MP induced cytotoxicity.

The main mechanism of 6-MP induced apoptosis appears to be a consequence of aberrant mismatch repair pathway signalling, as shown in primary $CD4^+$ T lymphocytes, through the activation of the intrinsic mitochondrial pathway [35]. We delineated the SAM-specific effects on cell proliferation, viability and apoptosis, and found that the presence of exogenous SAM decreases the extent of both cytostatic and cytotoxic effects of 6-MP (Fig. 2), as well as delaying the onset of 6-MP induced apoptotic signalling (Fig. 3). This led us to hypothesize that SAM does not have direct anti-apoptotic effects, but rather reverses the effects of 6-MP by altering the drug metabolism dynamics and, consequently, the amount of cytotoxic metabolites formed. We confirmed this notion by showing that addition of exogenous SAM to culture media significantly lowered the concentration of both cytotoxic TGN and MeTIMP metabolites (Fig. 4). Furthermore, the concentration of ATP was inversely correlated with MeTIMP concentration, indicating that the extent of MeTIMP induced inhibition of DNPS determines the concentrations of intracellular ATP, and consequently SAM (Fig. 5). Evidently, the addition of exogenous SAM results not only in less extensive DNPS inhibition due to lower MeTIMP production, resulting in less depleted ATP levels, but also limits the production of TGNs. Therefore, we propose that SAM must act as a positive modulator of TPMT activity, which results in a greater amount of 6-MP being converted to 6-MMP, a process that occurs more extensively than the HPRT catalyzed conversion to TIMP (Fig. 1). TPMT activity therefore determines the availability of TIMP for the multienzyme conversion to TGNs, as well as for the downstream TPMT-mediated MeTIMP synthesis.

The addition of exogenous SAM also reversed the 6-MP-induced decrease in intracellular SAM (Fig. 5B), TPMT activity and protein levels, but had no effect on TPMT mRNA expression (Fig. 6). Moreover, intracellular SAM levels correlated well with TPMT activity. Although we have not evaluated the effect of exogenous SAM on TPMT mRNA stabilisation or protein synthesis rate, we propose that SAM has a stabilizing effect on TPMT, and maintains its enzyme activity levels. This notion is supported by reports of a positive effect of SAM on in vitro intrinsic stability of the recombinant wild-type protein [15]. In addition, the binding of a SAM analogue, sinefungin, into the active site of the bacterial orthologue of TPMT facilitates conformational stabilisation of the enzyme, which prevents intracellular degradation [17]. We thus assume that the mechanism of SAM-induced TPMT activity involves the SAM-dependent post-translational stabilisation of TPMT. As a result of DNPS inhibition by MeTIMP, ATP is depleted, which prevents SAM re-synthesis through the trans-methylation pathway. This causes lower TPMT stability and activity and limits the deactivation of 6-MP to 6-MMP, leading to higher TGN production and cytotoxicity. However, in cells co-treated with 6-MP and SAM, the intracellular level of SAM remains sufficiently high to maintain high TPMT activity and protein level, resulting in rapid 6-MMP production, which in turn causes low availability of TIMP as a substrate for the production of cytotoxic metabolites.

Due to the pleiotropic properties of SAM, the principal methyl donor in processes such as nucleic acid, protein and phospholipid methylation [36], other mechanisms that may be responsible for the reversal of cytotoxic properties of 6-MP have to be considered. Due to SAM depletion, DNA was shown to be hypomethylated in MOLT cells treated with 6-MP [37,38], which may cause altered expression profiles of genes involved in cell proliferation and/or cell survival signalling. Although exogenous SAM might prevent DNA hypomethylation following treatment with 6-MP, which remains to be determined, this mechanism would only partly account for the reversal of cytotoxic effects in cells treated with both 6-MP and SAM, since it does not explain the significant decrease in the amounts of cytotoxic MeTIMP and TGNs observed in our study. Similarly, SAM-dependent polyamine synthesis most probably remains largely unaltered [39], due to the addition of SAM, ensuring normal polyamine homeostasis, and consequently less compromised growth of 6-MP treated cells. However, no evidence exists for the interaction between metabolic pathways of polyamines and thiopurines. Therefore, we may conclude that the decrease in cytotoxic 6-MP metabolites is the consequence of post-translational protein stabilization of TPMT by SAM, resulting in lower availability of TIMP as the substrate for conversion to cytotoxic metabolites. However, the observed reduction in 6-MP toxicity on the cellular level may be the result of additional direct and/or indirect actions of SAM, which remains to be addressed.

Several implications of the present study regarding thiopurine treatment are evident, although a well-designed in vivo approach should be carried out to confirm these findings. In patients receiving 6-MP, a decrease in TPMT activity may be expected after 6-MP administration, due to DNPS inhibition and decreased synthesis of the stabilising factor SAM. In patients with wild-type or heterozygous mutant TPMT genotypes, who exhibit high and intermediate TPMT activity, respectively, a decrease in the enzyme activity may result in an overproduction of TGNs, increasing the risk of undesirable toxicity. On the other hand, high levels of endogenous SAM, as well as potential compensatory responses to SAM depletion, may contribute to the detoxification of the drug, and consequently render the wild-type patients to be non-responders, by decreasing the production of cytotoxic TGNs. The availability of folate pools may also significantly influence 6-MP related cytotoxic effects, since the metabolic fluxes of homocysteine remethylation and downstream SAM synthesis are folate dependent [40]. The mechanism described in the present study could therefore play an important role in patients receiving folates in dietary supplements during thiopurine treatment, modulating the amount of SAM, and consequently TPMT activity. Further in vivo studies of the correlation of TPMT with the activity of enzymes involved in SAM metabolism (e.g. methionine adenosyltransferase, S-adenosylmethionine decarboxylase, 5,10-methylenetetrahydrofolate reductase), could reveal additional factors influencing treatment with 6-MP. Moreover, the presence of activity-modulating genetic polymorphisms in these enzymes may explain the poor TPMT genotype-to-phenotype correlations observed in some individuals [20,21].

In conclusion, our results provide new insights into the regulation of 6-MP metabolism by the TPMT-modulating factor SAM, and suggest that SAM status is an important parameter of thiopurine therapy-related toxicity.

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