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Differential role of thiopurine methyltransferase in the cytotoxic effects of 6-mercaptopurine and 6-thioguanine on human leukemia cells

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ABSTRACT

The thiopurine antimetabolites, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) are inactive prodrugs that require intracellular metabolism for activation to cytotoxic metabolites. Thiopurine methyltransferase (TPMT) is one of the most important enzymes in this process metabolizing both 6-MP and 6-TG to different methylated metabolites including methylthioinosine monophosphate (meTIMP) and methylthioguanosine monophosphate (meTGMP), respectively, with different suggested pharmacological and cytotoxic properties. While meTIMP is a potent inhibitor of *de novo* purine synthesis (DNPS) and significantly contributes to the cytotoxic effects of 6-MP, meTGMP, does not add much to the effects of 6-TG, and the cytotoxicity of 6-TG seems to be more dependent on incorporation of thioguanine nucleotides (TGNs) into DNA rather than inhibition of DNPS. In order to investigate the role of TPMT in metabolism and thus, cytotoxic effects of 6-MP and 6-TG, we knocked down the expression of the gene encoding the TPMT enzyme using specifically designed small interference RNA (siRNA) in human MOLT4 leukemia cells. The knock-down was confirmed at RNA, protein, and enzyme function levels. Apoptosis was determined using annexin V and propidium iodide staining and FACS analysis. The results showed a 34% increase in sensitivity of MOLT4 cells to 1 μM 6-TG after treatment with TPMT-targeting siRNA, as compared to cells transfected with non-targeting siRNA, while the sensitivity of the cells toward 6-MP was not affected significantly by down-regulation of the TPMT gene. This differential contribution of the enzyme TPMT to the cytotoxicity of the two thiopurines is probably due to its role in formation of the meTIMP, the cytotoxic methylated metabolite of 6-MP, while in case of 6-TG methylation by TPMT substantially deactivates the drug.

1. Introduction

The thiopurine antimetabolites, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) are purine nucleoside analogues and are currently the cornerstones in the treatment protocols of childhood acute leukemias [1–5]. These drugs have a relatively narrow therapeutic index and can result in life-threatening toxicity, mainly in the form of myelosuppression [1,6].

Both 6-MP and 6-TG undergo extensive metabolism (Fig. 1) before exerting cytotoxicity by incorporation into DNA as thioguanine nucleotides (TGNs) and in the case of 6-MP also inhibition of *de novo* purine synthesis (DNPS) pathway [7]. Methylation of 6-MP and 6-TG by the polymorphic enzyme thiopurine methyltransferase (TPMT) results in inactive metabolites methylmercaptopurine and methylthioguanine, respectively.

Although the mechanisms underlying resistance of leukemic cells towards 6-MP and 6-TG are not well understood [2], reduction in or lack of hypoxanthine–guanine phosphoribosyl transferase (HGPRT) activity and altered TPMT activity are suggested to be involved in sensitivity and resistance to these agents [8]. Besides, defects in DNA mismatch repair system is another known reason behind acquired resistance against several anti-malignancy drugs, including 6-MP and 6-TG [9,10].

Intracellularly, 6-MP is converted by HGPRT into 6-thioinosine-5′-monophosphate (TIMP) which can be converted further into 6-thioguanosine-5′-monophosphate (TGMP) involving two additional enzymes, inosine monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthetase (GMPS) [1,6]. This process is in competition with methylation by TPMT, which is influenced by common genetic polymorphisms in the TPMT gene [11]. On the other hand, 6-TG is directly converted by HGPRT into TGMP, which is then converted further to TGNs, which can be incorporated into RNA and DNA (Fig. 1).

The activity of TPMT is controlled genetically and has a trimodal distribution of activity [12–15]. A high degree of concordance has

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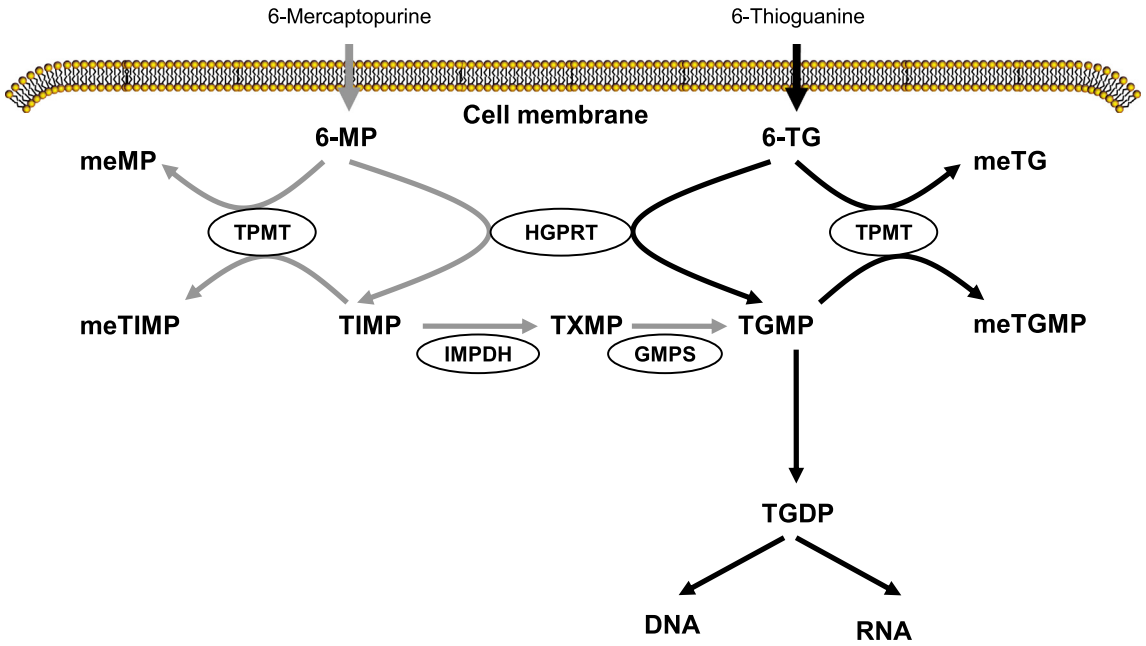

Fig. 1. Metabolism of the 6-MP and 6-TG by human leukemia cells. Intracelluarly 6-TG is converted directly by HGPRT to TGMP, 6-MP is converted first to TIMP by HGPRT then to TXMP by IMPDH and finally to TGMP by GMPS. Both 6-MP and 6-TG and their respective monophosphates (TIMP and TGMP) are further metabolized to methylated metabolites inside the cell by TPMT.

been demonstrated between TPMT genotype and phenotype in Caucasians [16] and erythrocyte TPMT activity correlates with TPMT in other cells and tissues and is a convenient assay for enzyme activity [6].

In case of 6-MP, an active metabolite, methylthioinosine monophosphate (meTIMP) is produced from TIMP by TPMT which inhibits *de novo* purine biosynthesis in concentrations that exceed those of TGNs [12]. It has been shown that TPMT activity is inversely related to the concentration of active thioguanine metabolites after administration of thiopurines [17].

Since 6-TG is directly converted into TGMP in a single-step reaction catalyzed by HGPRT, it is suggested that 6-TG may have an advantage over 6-MP [18]. However a randomized clinical study concluded that, as compared with 6-MP, 6-TG causes excess toxicity without an overall benefit and 6-MP should therefore remain the thiopurine of choice for continuing therapy of childhood lymphoblastic leukemia [19].

The major difference between the metabolisms of the two drugs is that 6-TG forms TGNs directly while 6-MP forms intermediate metabolites which are major substrates for TPMT and the resulting methylated metabolites are produced at the expense of TGNs (Fig. 1). Thus, intracellular TGNs seem to be formed more reliably after 6-TG is given than after 6-MP is given, especially in patients with high TPMT activity [19]. As a matter of fact, results of clinical studies have shown higher levels of TGNs in patients treated with 6-TG compared to 6-MP [7].

It has been suggested that DNPS inhibition by meTIMP significantly contributes to the cytotoxic action of 6-MP [20], however, the equivalent of meTIMP in 6-TG metabolism, namely, methylthioguanosine monophosphate (meTGMP) is not known to be as cytotoxic as meTIMP.

Coulthard et al. demonstrated a 4.4-fold increase in sensitivity to 6-MP, a rise in intracellular levels of meTIMP, and a decrease in levels of DNA-TGN after induction of TPMT activity in the ecdysone receptor 293 embryonic kidney cell line. Conversely, induction of TPMT produced a 1.6-fold decrease in sensitivity to 6-TG, a decrease in levels of DNA-TGN, and an increase in levels of methylated thioguanosine monophosphate [20]. Moreover, Dervieux et al. demonstrated that human CCRF-CEM cell lines that overexpress TPMT were more sensitive to 6-MP and less sensitive to 6-TG than cells not overexpressing TPMT [21].

Recently, Misdaq et al. established a TPMT knock-down cell culture model which mimics human TPMT deficiency polymorphism by using Jurkat cells and they concluded that TPMT affects 6-TG and 6-MP differently [22].

To further study the role played by TPMT in the intracellular metabolism of 6-MP and 6-TG by leukemic cells and contribution of methylated metabolites on the viability of these cells, we silenced human TPMT gene in MOLT4 leukemia cells with TPMT-targeting siRNA and confirmed the down-regulation of the gene encoding for this enzyme by measuring its messenger RNA (mRNA), protein, and enzyme activity levels and compared them with their levels in non-transfected cells and cells transfected with non-targeting siRNA. Subsequently, we performed cytotoxic assays with both drugs based on annexin V and propidium iodide staining, employing flow cytometry. To our knowledge, this is the first time that siRNA silencing of TPMT is employed to study the contribution of this gene to cytotoxic effects of thiopurines.

2. Materials and methods

2.1. Chemicals

6-Mercaptopurine, 6-thioguanine (Sigma–Aldrich, Stockholm, Sweden); L-glutamine, and penicillin–streptomycin (Life Technologies, Paisley, United Kingdom); TaqMan reagents and gene expression assays (including the assays for TPMT (No. Hs00909010_g1) and gylceraldehyde-3-phosphate dehydrogenase (GAPDH) (No. Hs99999905_m1)) (Applied Biosystems, Stockholm, Sweden); and TPMT siGENOME SMARTpool siRNA (Dharmacon Research, Inc., Lafayette, CO) were purchased from the sources indicated.

2.2. Cell line

The acute T-lymphoblastic leukemia MOLT4 cell line (obtained from American Type Culture Collection, Rockville, MD) was

subcultured at 37 °C in RPMI-1640 medium supplemented with fetal calf serum (FCS) (10%), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) in humidified incubator containing 5% CO_2. Cells (approximately 0.8–1.5 × 10^6 cells/ml) were used for experiments during the logarithmic phase of their growth.

2.3. Transfection of TPMT gene with siRNA in MOLT4 Cells

Prior to siRNA transfection cells were washed in PBS and resuspended in 400 μl volume of RPMI-1640 followed by addition of siRNA at a final concentration of 100 nM targeting the TPMT gene or of non-targeting siRNA at room temperature in 0.4 cm electroporation cuvettes using an electroporator (Gene Pulser Xcell Electroporation System, Bio-Rad).

The mixture was pulsed at 340 V for 10 ms, repeated once after 24 h [8,23].

Cells were transferred from the cuvettes to pre-heated (37 °C) post-electroporation medium containing RPMI-1640, L-glutamine (2 mM) and 20% FCS in 25 cm^2 tissue culture flasks.

Small interfering RNAs used were the siGENOME® siRNA against human TPMT and non-targeting siGENOME® siRNA (negative control) not binding to any human mRNA sequences for the control cells.

A non-transfected control (wild-type MOLT4) sample was always included in parallel with the rest of the samples to show the effect of siRNA transfection *per se* on the cells and the cytotoxicity of the drugs used during the experiments.

Transfection efficiency was evaluated by measuring TPMT mRNA levels 24 h, 48 h and 72 h after transfection to study if and when an acceptable level of down-regulation is achieved.

Antibiotics were avoided during electroporation since the intracellular concentration of antibiotics may reach high and potentially toxic levels if allowed to flow into the open pores of electroporated cells and the recovery medium used after electroporation contained 20% FCS to increase the down-regulation and enhance cell viability [23].

2.4. RNA Extraction and cDNA synthesis

Total RNA was extracted by the RNeasy procedure (RNeasy Mini Handbook; Qiagen, KEBO Lab, Sweden) according to the manufacturer's instructions. RNA concentrations and quality were determined using a NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE), RNA with an OD ratio of 1.99–2.0 at 260/280 was considered acceptable.

Complementary DNA was produced using Invitrogen SuperScript™ III first strand synthesis system according to the manufacturer's instructions.

2.5. mRNA expression of TPMT in MOLT4 cells by RT-PCR

The cDNA samples were used as templates whereas an endogenous housekeeping gene (GAPDH) was quantified as a positive control and used for normalization of the different template values. Real-time PCR reactions were carried out using the 7500 real-time PCR system and reagents from Applied Biosystems (Foster City, CA).

Each real-time TaqMan PCR reaction mixture (20 μl) contained 9 μl cDNA template, 1 μl TaqMan® Gene Expression Assay and 10 μl TaqMan Universal PCR Master Mix (according to manufacturer's instruction "TaqMan® Gene Expression Assays" Applied Biosystems).

The mRNA expression of TPMT in transfected cells was related to the expression in cells treated with the non-targeting negative control siRNA.

2.6. Western blotting

Cells were washed with PBS and lysed in IPB-7 (1 mg/mL phenylmethylsulfonyl fluoride, 0.1 mg/mL trypsin inhibitor, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mmol/L Na_3VO_4, and 1 mmol/L NaF). The samples were subjected to SDS–PAGE and thereafter blotted onto a PVDF membrane (Bio-Rad). The protein bands were probed using antibodies against Actin and TPMT, from Santa Cruz Biotechnology. (Montgomery, TX. Proteins were visualized with the ECL procedure (Amersham Biosciences). The Western blotting results were analyzed with NIH Image 1.62 software.

2.7. TPMT activity measurement

The TPMT activity was determined by measuring the 6-meMP formed in the enzymatic reaction by Liquid chromatography–mass spectrometry (LC–MS) method which is based on the conversion of 6-MP to 6-meMP with S-adenosyl-L-methionine (SAM) as methyl donor.

MOLT4 cell lysates were prepared and enzyme activity assay was carried out according to an assay modified in our laboratory from a previously described method for TPMT phenotyping by Ford et al. [24]. Results were expressed as units per five million MOLT4 cells, where one unit of enzyme activity represents the formation 1 nmol of 6-meMP per hour.

Standard curve was made from cells of 2, 5 and 10 millions suspended in eppendorf tubes containing 200 μL cold dilute saline (0.9 g/L sodium chloride in water), the mixture was vortexed and sonicated on ice. The samples were kept at −80 °C until analysis.

2.8. Cytotoxicity assays

After siRNA transfection, the MOLT4 cells were allowed to recover for 24 h before initiating the experiments, and the cells were incubated with different concentrations of both 6-MP and 6-TG for the durations of 24 h, 48 h and 72 h in 24-well tissue culture plates.

2.9. Annexin V and propidium iodide flow cytometry apoptosis analysis

Cells were washed in PBS and resuspended in 100 μl of binding buffer (10 mM HEPES, 0.9% NaCl, 2.5 mM $CaCl_2$, 0.1% BSA) containing 5 μl of phycoerythrin-conjugated Annexin-V (Annexin-V-PE; PharMingen, San Diego, CA) and incubated at room temperature in the dark for 15 min, followed by the addition of 500 μl of binding buffer containing 5 μl of propidium iodide. Apoptosis following drug treatment was assessed by flow cytometry (Becton Dickinson, San Jose, CA) using annexin V and propidium iodide staining, the cells that had been transfected with non-specific siRNA were used as controls.

2.10. Statistical analysis

The data were analyzed with Student's *t* test and *P* values <0.05 were considered statistically significant.

3. Results

3.1. Transfection with siRNA and TPMT down-regulation

To evaluate the role of TPMT in the metabolism of 6-MP and 6-TG, we transfected MOLT4 cells with siRNA against TPMT. Different

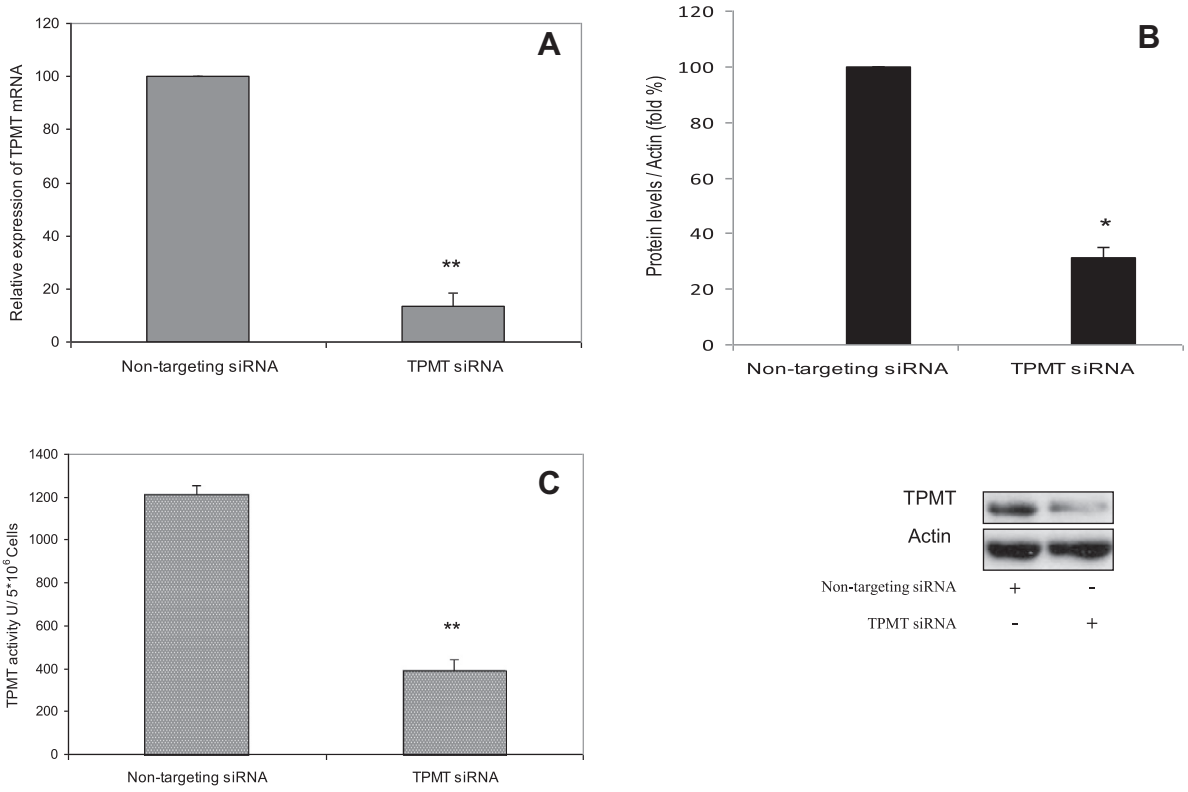

Fig. 2. (A) TPMT mRNA level in cells transfected with siRNA targeting this gene, compared with cells transfected with non-targeting siRNA, as determined by RT-PCR analysis. TPMT mRNA was expressed as percentage of the non-transfected cells. GAPDH was used as internal standard. Data are presented as mean ± SD of three independent experiments. $^{**}p < 0.01$. (B) Western blot analysis of TPMT knock-down cells. Data are presented as mean ± SD of three independent experiments. $^{*}p < 0.05$. (C) The TPMT enzyme activity in MOLT4 cells treated with siRNA targeting the enzyme as compared to cells transfected with non-targeting siRNA. The TPMT activity expressed in nmol/l 6-meMP. Data are presented as mean ± SD of three independent experiments. $^{**}p < 0.01$.

time points, 24 h, 48 h and 72 h were tested in order to evaluate the efficiency of electroporation process.

TPMT mRNA levels in siRNA-treated MOLT4 cells showed an 86% reduction 48 h after transfection, in comparison with cells treated with non-targeting siRNA (Fig. 2A). TPMT mRNA levels were unchanged in non-targeting siRNA-treated MOLT4 cells compared with TPMT mRNA levels in non-treated wild-type MOLT4 cells (data not shown).

The knock-down of the TPMT gene was further confirmed at the protein level 48 h after transfection by Western blotting (Fig. 2B) and showed a significant reduction (70%).

Moreover, results of the enzyme activity as determined by LC–MS correlated well with the results of Western blotting and RT-PCR and showed a 71% reduction in TPMT enzyme activity in MOLT4 cells 48 h post-transfection with siRNA targeting the enzyme, as compared with, cells treated with non-targeting siRNA (Fig. 2C).

3.2. Cytotoxicity assays and flow cytometric analysis

The MOLT4 cells were incubated with a range of concentrations of either 6-MP and 6-TG for a period of 48 h under a humidified atmosphere containing 5% CO_2 at 37 °C. To measure total cell death, cells were stained with annexin V and propidium iodide and analysed by flow cytometry. Then cell viabilities were determined and expressed as percentage of the viable control (non-drug treated) cells. Double negative (annexin V and PI negative) cells were considered as viable.

We chose 48 h incubations which is consistent with the delayed effects of thiopurines [21].

Both transfected (TPMT-siRNA transfected and non-targeting-siRNA transfected) and non-transfected MOLT4 cells were more sensitive to 6-TG than to 6-MP. The sensitivity of TPMT-siRNA transfected MOLT4 cells to 6-TG was significantly higher compared to the non-targeting-siRNA transfected MOLT4 cells. The cells transfected with TPMT siRNA were 34%, 23% and 13% more sensitive to cytotoxic effects of 6-TG at concentrations of 1, 2, and 3 μM, respectively (Fig. 3). However, transfection with TPMT-siRNA did not result in any significant alteration to the cytotoxic effects of 6-MP, compared to transfection with non-targeting siRNA (Fig. 3).

We investigated the effect of electroporation/transfection *per se* on the cells and their sensitivity toward the agents used in this work by studying the cytotoxicity of daunorubicin (DNR) on the MOLT4 cells employing annexin V and propidium iodide staining with FACS analysis. As we expected, after 48 h incubation, there was no alteration in sensitivity between the non-transfected cells (wild-type MOLT4), cells transfected with non-targeting siRNA, and cells transfected with siRNA against TPMT (Fig. 4).

4. Discussion

Although the thiopurines 6-MP and 6-TG are well known drugs for the treatment of leukemia, their main mode of action is still not well understood.

Inside cells, 6-MP and 6-TG are converted by HGPRT into TIMP and TGMP, respectively, and subsequently to the active cytotoxic TGNs, which are incorporated into DNA in the reduced form. TIMP and TGMP are methylated by TPMT an S-adenosylmethionine-dependent cytosolic enzyme, into meTIMP and meTGMP, respectively [21]. However, the potential contributions of meTGMP and meTIMP to the pharmacological properties of 6-TG and 6-MP, respectively, remain unclear [21].

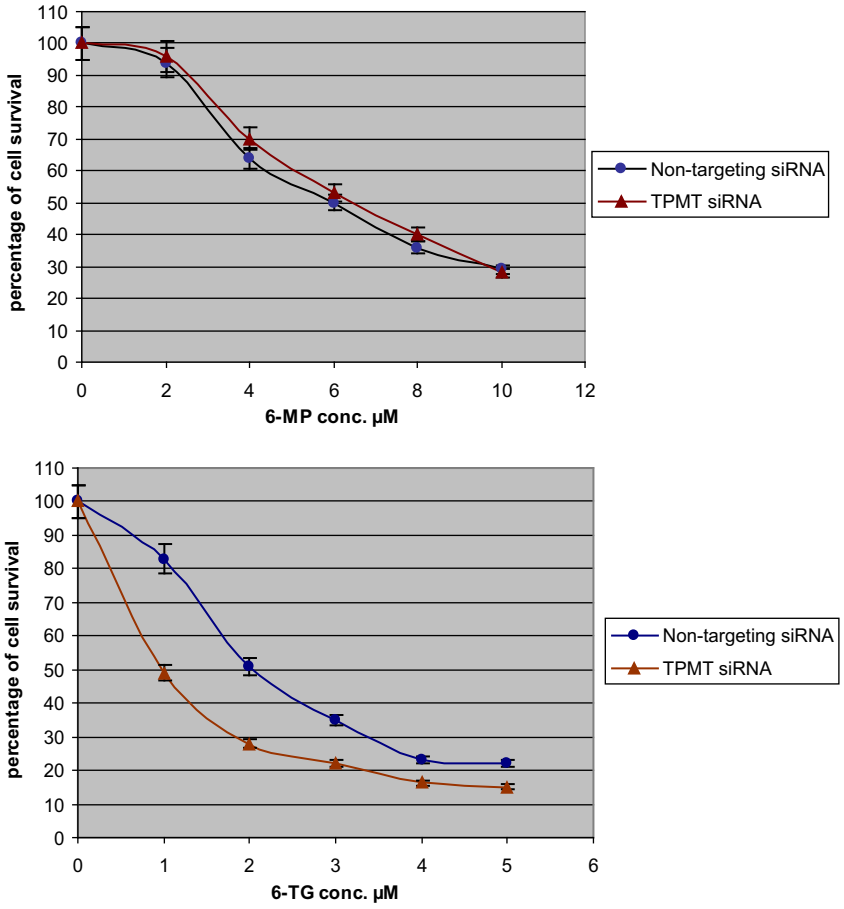

Fig. 3. Cytotoxicity assay with annexin V and propidium iodide staining showing that cells are more sensitive to 6-TG when they have been transfected with TPMT siRNA, compared to cells transfected with non-targeting siRNA. Cell viabilities were determined and expressed as percentage of the viable control (non-drug treated) cells. Data are presented as mean ± SD of three independent experiments.

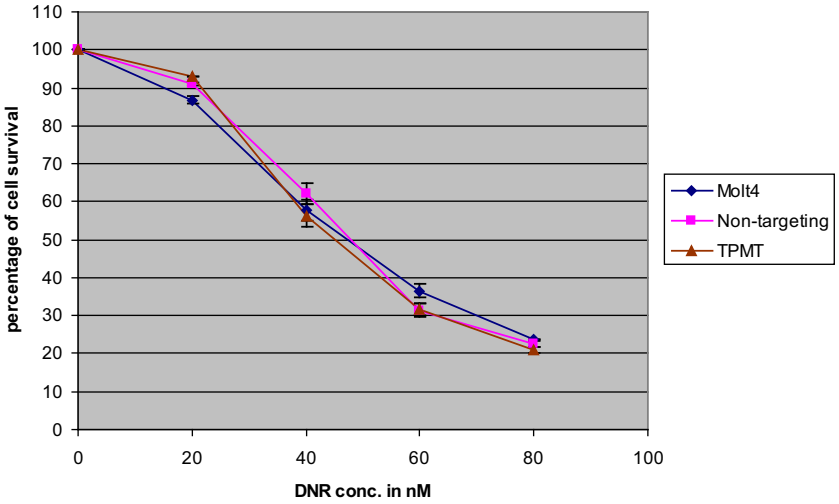

Fig. 4. Effect of transfection *per se* on cytotoxicity of daunorubicin on MOLT4 cells, as determined by annexin V and propidium iodide staining. Data are presented as mean ± SD of three independent experiments.

Previous studies have suggested that although both 6-TG and 6-MP exert cytotoxic effect through incorporation of TGNs into newly synthesized DNA, yet another important way of action of 6-MP is inhibition of DNPS through the production of meTIMP by TPMT, which is not produced in cells exposed to 6-TG [20].

It has been shown that at equimolar concentrations, 6-TG is considerably more cytotoxic than 6-MP in different human leukemic cell lines [18] and patients with high functional TPMT activity may convert the majority of 6-MP into methylated metabolites with inadequate production of 6-TGNs. The higher toxicity of 6-

TG is attributed to its rather direct conversion into TGNs and bypassing some enzymatic checkpoints that are present with 6-MP metabolism [4,22].

To identify patients with low TPMT activity who have an increased risk of toxicity if treated with standard doses of these medications, phenotyping in red blood cells and/or genotyping of TPMT is performed routinely in many medical centers before starting thiopurine treatment.

It has been shown that methylation of 6-MP contributes to the antiproliferative properties of the drug, probably through inhibition of *de novo* purine synthesis by methylmercaptopurine nucleotides, whereas thioguanine is inactivated primarily by TPMT [21]. Taking these to consideration, the metabolite profile can have a great impact on the clinical outcome and toxicity of the thiopurine drugs.

In two *in vitro* studies performed on CCRF-CEM and embryonic kidney cells, increased TPMT activity was associated with higher levels of methylated metabolites and lower levels of TGNs, resulting in elevated sensitivity to 6-MP and reduced sensitivity to 6-TG [20,21]. However, a recent study by Misdaq et al. which is technically closer to our work, through transfection of a TPMT-specific short hairpin RNA (shRNA) expressing plasmid in T-lymphocytes, showed that cells with downregulated TPMT activity were more sensitive to high concentrations of 6-MP (10 μM/L) and 6-TG (8 μM/L) than wild type cells [22].

Interestingly, the same study showed that responses to 6-MP treatment were not much affected by TPMT status in wild type and knock-down cells when considering the IC_{60} concentrations for wild type and knock-down cells, 4.6 μM/L and 4.7 μM/L, respectively.

While they concluded that 6-TG activity was more affected by TPMT levels than 6-MP as reflected by IC_{60} concentrations for wild type and knock-down cells, 2.7 μM/L and 0.8 μM/L, respectively.

In this study, we investigated the role of TPMT in thiopurine toxicity in cultured human MOLT4 cells which are known to be sensitive to thiopurines and they express TPMT activity [2]. Since knock-down models are more representative of the clinical picture of TPMT gene polymorphisms than the overexpression ones, we employed highly specific siRNA to downregulate this gene in human T-lymphoblastic leukemia cells to investigate the potential contribution of TPMT in metabolism and thus, cytotoxicity of the thiopurines.

We chose 48 h treatment because these agents are slow acting and 24 h was too short time to detect any significant cell death, on the other hand spontaneous apoptosis can be expected in cases of 72 h or longer incubations.

The downregulation of TPMT enzyme was confirmed at RNA, protein, and enzymatic function levels.

As expected, 6-TG was found to be more potent, in terms of cytotoxicity, than 6-MP in both siRNA treated and non-treated wild type cells, which could be related to the relatively direct metabolism of 6-TG in its way to TGNs as compared to 6-MP, which passes through many rate-limiting steps [4,7].

There was a significant increase in sensitivity to 6-TG on down-regulation of the TPMT gene as measured by annexin V and propidium iodide staining at wide range of concentrations of the drug (Fig. 3), with the highest increase (34%) at 1 μM concentrations. These results are in line with the previously published results, that TPMT gene status mostly affects response to 6-TG rather than 6-MP. Conversely, when the cells were treated with 6-MP, there was no significant change in the sensitivity to 6-MP in cells with down-regulated TPMT gene.

We realize that the cell line (MOLT4), the knock-down technique (siRNA) we applied in this work, and the extent of down regulation are different from the ones by Misdaq et al. and these might explain why they had an altered to response to 6-MP at higher concentration of (10 μM/L) following transfection.

To our knowledge, this is the first knock-down technique employing highly specific siRNA to study the potential contribution of TPMT in the metabolism of thiopurines in human MOLT4 leukemia cells after significant down-regulation of the enzyme which reflects its genetic polymorphism.

Our results indicate that TPMT metabolizes 6-MP and 6-TG differently and provide more insights into the role of these methylated metabolites and suggest potential cytotoxic effects of these metabolites including meTIMP which is produced by TPMT in case of incubation of the cells with 6-MP, while its counterpart meTGMP, in case of treating the cells with 6-TG, does not show the same significant cytotoxic effects. This can explain why we did not observe more tolerance to cytotoxic effects of 6-MP after treatment with TPMT-targeting siRNA and blockage of methylation pathway which leads to production of higher amounts cytotoxic TGNs. As a matter of fact, the idea that inhibition of DNPS is minimum with 6-TG is further supported by the fact that meTGMP is a twelve-fold less potent inhibitor of the DNPS than meTIMP [25,26].

We concluded that targeting TPMT gene by siRNA can be helpful in understanding possible clinical consequences of TPMT polymorphism. In addition, we showed that TPMT provides a differential contribution to the cytotoxic effects of the two drugs through methylation, by converting 6-MP to metabolites with remarkable cytotoxic effects, while methylation of 6-TG substantially decreases cytotoxicity of this agent.

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