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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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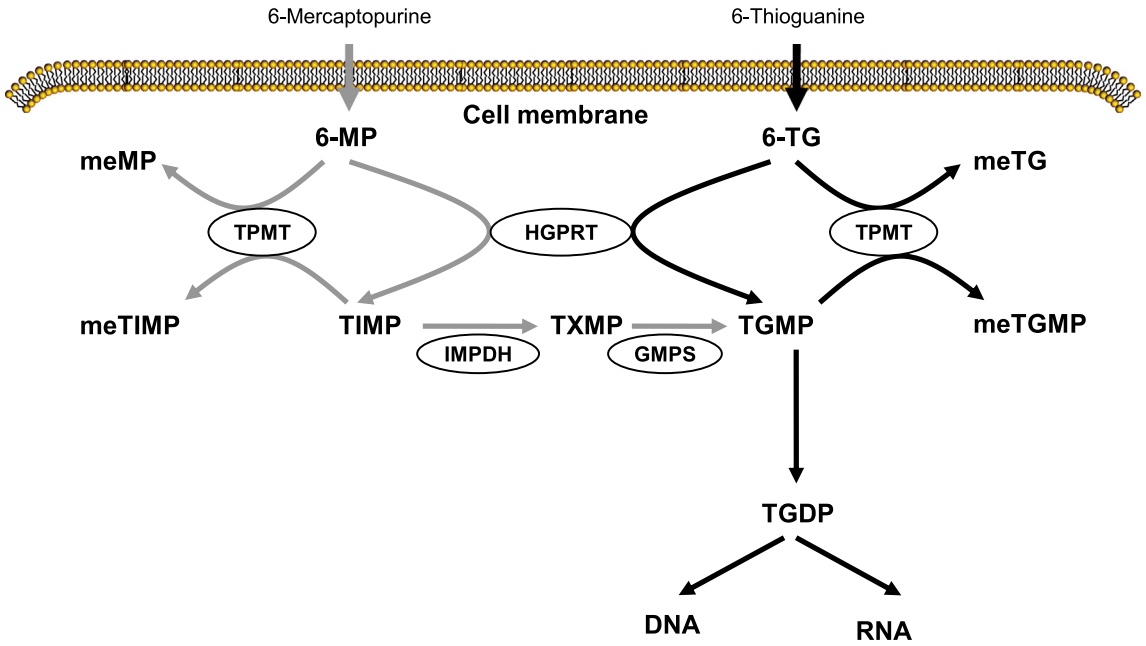

Fig. 1. Metabolism of the 6-MP and 6-TG by human leukemia cells. Intracelluarly 6-TG is converted directly by HGPRT to TGMP, 6-MP is converted first to TIMP by HGPRT then to TXMP by IMPDH and finally to TGMP by GMPS. Both 6-MP and 6-TG and their respective monophosphates (TIMP and TGMP) are further metabolized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