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Involvement of the concentrative nucleoside transporter 3 and equilibrative nucleoside transporter 2 in the resistance of T-lymphoblastic cell lines to thiopurines ☆

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Abstract

Mechanisms of resistance to thiopurines, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) were investigated in human leukemia cell lines. We developed two 6-MP- and 6-TG-resistant cell lines from the human T-lymphoblastic cell line (MOLT-4) by prolonged exposure to these drugs. The resistant cells were highly cross resistant to 6-MP and 6-TG, and exhibited marked reduction in cellular uptake of 6-MP (70% and 80%, respectively). No significant modification of the activities of hypoxanthine–guanine phosphoribosyl transferase, thiopurine methyltransferase or inosine monophosphate dehydrogenase was observed. Real-time PCR of concentrative nucleoside transporter 3 (CNT3) and equilibrative nucleoside transporter 2 (ENT2) of resistant cells showed substantial reductions in expression of messenger RNAs. Small interfering RNA designed to silence the CNT3 and ENT2 genes down-regulated the expression of these genes in leukemia cells. These decreases were accompanied by reduction of transport of 6-MP (47% and 21%, respectively) as well as its cytocidal effect (30% and 21%, respectively). Taken together these results show that CNT3 and ENT2 play a key role in the transport of 6-MP and 6-TG by leukemia cells. From a clinical point of view determination of CNT3 and ENT2 levels in leukemia cells may be useful in predicting the efficacy of thiopurine treatment.

Keywords: 6-Mercaptopurine; 6-Thioguanine; Resistance; Leukemia; Transport

6-Mercaptopurine (6-MP) and 6-thioguanine (6-TG) are important agents for the induction and maintenance of remissions in patients with acute myelocytic and lymphocytic leukemia [1]. These compounds are inactive prodrugs and their cytotoxicity is exerted by active metabolites formed intracellularly [2,3]. 6-MP is metabolized by at least three different pathways (Fig. 1).

The mechanisms underlying the resistance of leukemic cells to 6-MP and 6-TG are not well understood. The most extensively characterized mechanism is a reduction in or lack of hypoxanthine–guanine phosphoribosyl transferase (HGPRT) activity [4]. In addition, altered thiopurine methyltransferase (TPMT) activity can influence the degree of sensitivity to 6-MP and 6-TG [5]. Moreover, resistance to a wide range of anti-malignancy drugs, including 6-MP and 6-TG, can also be acquired through loss of DNA-mismatch repair activity [6–8]. Resistance to nucleoside analogs as a result of defective nucleoside transporter-mediated uptake of nucleoside analogs has also been described [9–13]. These transporters can be divided on the basis of their mechanism of

☆ *Abbreviations:* 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; HGPRT, hypoxanthine–guanine phosphoribosyl transferase; IC_{50}, the drug concentration inhibiting cell growth by 50%; IMPDH, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase; TPMT, thiopurine methyltransferase; meMPR, methyl mercaptopurine riboside; CNT3, concentrative nucleoside transporter 3; ENT2, equilibrative nucleoside transporter 2; TGMP, 6-thioguanosine 5′-monophosphate; TIMP, 6-thioinosine 5′ monophosphate; MRP(s), multi-drug resistance-associated protein(s); NT(s), human nucleoside transporter(s).

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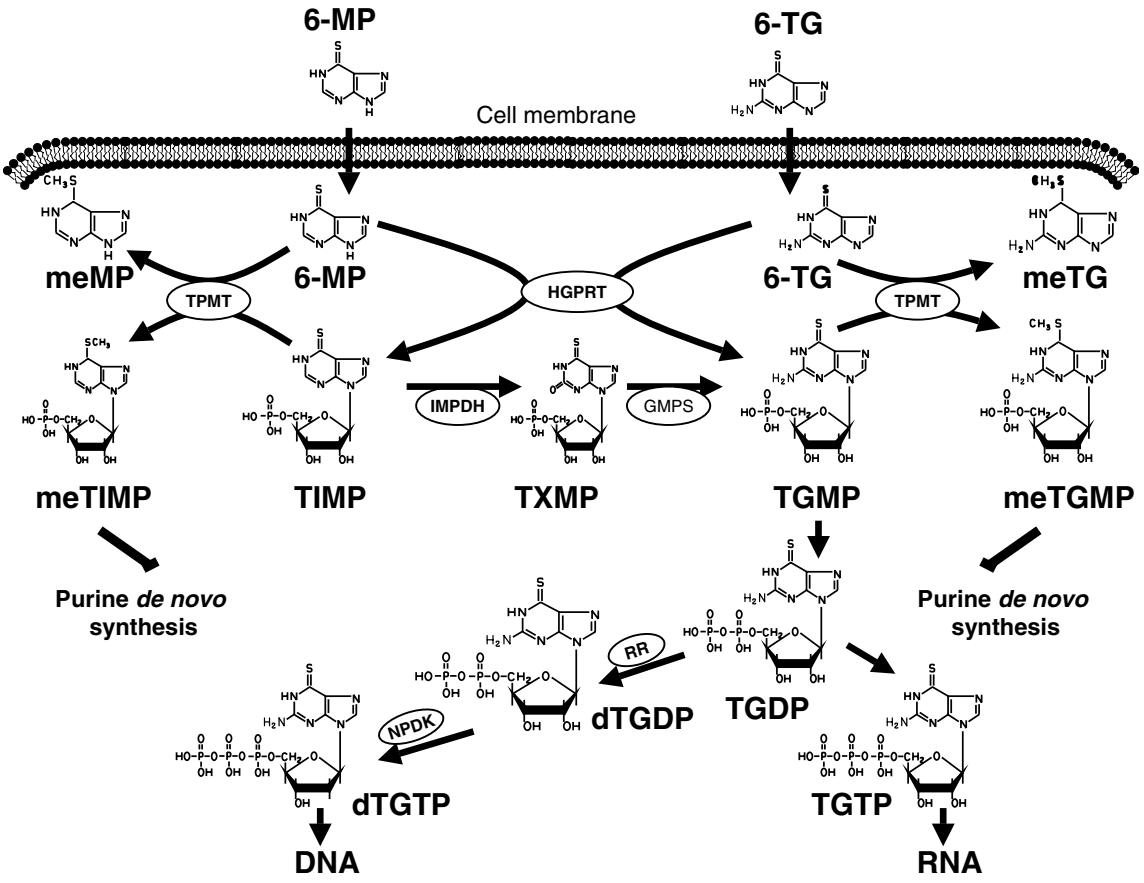

Fig. 1. Metabolism of the thiopurines, 6-mercaptopurine (6-MP), and 6-thioguanine (6-TG), by mammalian cells. 6-MP is metabolized via three different pathways: phosphorylation to thioguanine nucleotides by hypoxanthine–guanine phosphoribosyl transferase (HGPRT), inosine monophosphate dehydrogenase (IMPDH), and guanosine monophosphate synthetase (GMPS); and methylation by thiopurine methyltransferase (TPMT) to inactive methyl mercaptopurine (meMP) and methylthioinosine monophosphate (meTIMP). 6-TG is phosphorylated directly into 6-thioguanosine 5′-monophosphate (TGMP) through the enzyme HGPRT. 6-TG can also be methylated by TPMT to produce inactive methyl thioguanine (meTG). 6-MP and 6-TG can also be converted to inactive thiouric acid, that is modulated by the enzyme xanthine oxidase. As the leukemic cells almost lack the xanthine oxidase activity, this pathway is not the case here and not illustrated. TIMP, 6-thioinosine 5′-monophosphate; RR, ribonucleotide reductase; NDPK, nucleoside diphosphate kinase; TXMP, 6-thioxanthosine 5′-monophosphate; TGDP, 6-thioguanosine 5′-diphosphate; TGTP, 6-thioguanosine 5′-triphosphate; dTGDP, deoxy-6-thioguanosine 5′-diphosphate; dTGTP, deoxy-6-thioguanosine 5′-triphosphate; meTGMP, *S*-methylthioguanosine 5′-monophosphate.

action into two major classes, equilibrative and concentrative [14]. Equilibrative (facilitated) transport mediates the net flux of nucleoside molecules across the plasma membrane only down a concentration gradient; whereas in the case of concentrative or Na^+-dependent transport by the SLC28 family, an electrochemical ion gradient drives active cellular uptake of nucleosides even against a concentration gradient. The human equilibrative family (SLC29) of proteins contains four members. ENT1 and ENT2 are extensively studied and were shown to have similar broad substrate specificities for purine and pyrimidine nucleosides, and ENT2 has ability to transport nucleobases as well [15]. ENT1 can be potentially inhibited by nanomolar concentrations of nitrobenzylthioinosine (NBTI) and dipyridamole, but cannot inhibit ENT2 efficiently even at higher NBTI concentration [14]. The concentrative nucleoside transporter family (SLC28) consists of three subtypes of sodium-dependent transporters; CNT1 is pyrimidine nucleoside preferring, CNT2 is purine nucleoside preferring, and CNT3 transports both pyrimidine and purine nucleosides across the cell membranes [16].

In the present study, we have examined the mechanisms underlying the resistance of the human acute T-lymphoblastic leukemia, MOLT4 cell line to 6-MP and 6-TG. We demonstrate here for the first time that reduced influx of 6-MP into leukemic cells as a result of low gene expression of CNT3 and ENT2 confers resistance to thiopurines. In order to further confirm this finding, we investigated the effects of double-stranded siRNAs targeting the CNT3, ENT1, and ENT2 genes. Taken together, our results indicate that low levels of CNT3 and ENT2 expression are, indeed, responsible for the resistance of MOLT4 cells to 6-MP, thereby elucidating a novel mechanism by which cells can become resistant to thiopurines.

Material and methods

Chemicals. 6-Mercaptopurine, 6-thioguanine, and their metabolites methylmercaptopurine riboside, *S*-adenosyl-L-methionine, 5-phosphoribose-1-pyrophosphate, hypoxanthine, inosine 5′-monophosphate (IMP), xanthosine 5′-monophosphate, *S*-(4-nitrobenzyl)-6-thioinosine, and dipyridamole (Sigma–Aldrich, Stockholm, Sweden); Tris–HCl, ammonium dihydrogen phosphate ($NH_4H_2PO_4$), and ethylenediaminetetraacetic acid (EDTA) (Merck, Darmstadt, Germany); [8-^{14}C]6-mercaptopurine (51 mCi/mmol); [8-^{14}C]inosine 5′-monophosphate (55 mCi/mmol) (Moravek Biochemicals, CA); [2,8-^{3}H]hypoxanthine (21.5 Ci/mmol) (Amersham, Little Chalfont, UK); TaqMan reagents and gene expression assays including the assays for CNT1, CNT2, CNT3, ENT1, ENT2, MRP4, MRP5, and GAPDH (with product numbers: Hs00188418_m1, Hs00188407_m1, Hs00223220_m1, Hs00191940_m1, Hs00155426_m1, Hs00195260_m1, Hs00194701_m1, and Hs99999905_m1) (Applied Biosystems, Stockholm, Sweden), and CNT3, ENT1 and ENT2 *SMART*pool siRNA duplexes (proprietary target sequences) (Dharmacon Research, Inc., Lafayette, CO) were purchased from the sources indicated.

Cell cultures. The MOLT4 cell line, derived from an acute T-lymphoblastic leukemia patient, was obtained from the American Type Culture Collection (Rockville, MD). The cells were subcultured twice weekly in RPMI-1640 medium supplemented with fetal calf serum (10%), penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM) at 37 °C under an atmosphere of humidified air containing 5% CO_2. 6-MP- and 6-TG-resistant sub-clones were selected from the original parent MOLT4 cell line by increasing the concentrations of these compounds in the culture medium stepwise until the final concentration of 5 μM was reached. The resistant cells were then cultured in drug-free medium for at least three passages before using them during logarithmic phase growth (approximately 0.8–1.5×10^6 cells/mL) for the experiments. In both resistant sublines, resistance was stable in the absence of drugs for at least 20 passages. The cells were counted employing coulter Multisizer (Coulter Electronics, Luton, UK).

Assaying cytotoxicity. The cytotoxicity study was performed using MTT assay, by treating log phase MOLT4 cells with different concentrations of drugs for 72 h, as described by Mosmann [17]. IC_{50} values were defined as the drug concentrations at which cell growth was inhibited by 50% in comparison to controls.

Transport assay. Initial transport and intracellular accumulation of 6-MP were measured by incubating logarithmically growing cells in the presence of [^{14}C]6-MP. Briefly, the cells were collected by centrifugation, washed and resuspended in Hepes-buffered RPMI-1640 medium (2×10^6 cells/mL), and incubated with 10 μM [^{14}C]6-MP at 37 °C from 10 s to 60 min. Uptake of 6-MP was terminated at the various time-points by the addition of ice-cold PBS, followed by centrifugation for 5 min at 1800*g* at 4 °C and then washing three times with ice-cold PBS. The cell pellets were resuspended in 150 μl ethanol 70%, and then stirred and centrifuged for 10 min at 4000*g*. The radioactivity in resulting protein-free supernatant was determined by liquid scintillation counting.

HGPRT, IMPDH, and TPMT enzyme assays. HGPRT assay was performed according to Pieters et al. [18] using 46.5 μM [^{3}H]hypoxanthine (with a specific radioactivity of 21.5 Ci/mM) and 2.25 mM 5-phosphorybose-1-pyrophosphate (PRPP) as substrates, and monitoring their conversion to inosine monophosphate (IMP). Inosine monophosphate dehydrogenase (IMPDH) activity was determined as described by Proffitt et al. [19] utilizing 250 μM [8-^{14}C]inosine 5′-monophosphate with a specific radioactivity of 500–1000 cpm/pmol as substrate and measuring the amount of xanthosine 5′-monophosphate produced. The TPMT assay was a modification of the radiochemical procedure developed by Weinshilboum et al. [20] in which non-radioactive *S*-adenosyl-L-methionine (SAM) was used as the methyl group donor, 10 μM 6-MP used as substrate, and the rate of conversion to 6-meMP monitored. In all three assays, HPLC was employed to quantitate the products, as described below. The protein contents of cell extracts were determined according to the method of Lowry et al. [21] (DC Protein Assay; Bio-Rad Laboratories, Hercules, CA).

High performance liquid chromatography system. The HPLC system consisted of a CM4000 pump (Milton Roy, LDC Division, USA), a CMA-240 autosampler (Carneige Medicine, Stockholm, Sweden) equipped with flow scintillation analyzer 500TR (Packard, Illinois, USA). All the mobile phases were filtered (0.22 μm filter Millipore, Ireland) and degassed.

$NH_4H_2PO_4$ 37.5 mM with 20% methanol (pH 3.37) was used as mobile phase to separate 6-MP and meMP. The separation column was a Hypersil Duet C18/SAX Column with diameter and particle size of 250×4.6 mm, 5 μ. The detection wavelength was set up at 290-nm absorbance. Mobile phase and scintillation fluid flow rates were set up at 2 and 6 mL/min, respectively. Both retention time and UV spectra were considered for identification of the peaks. The separation of IMP and XMP was done on a Whatman Particil 10 SAX column, and 100 mM $NH_4H_2PO_4$, pH 3.3, used as the mobile phase. The elution was carried out at a flow rate of 2 mL/min. Hypoxanthine and IMP were separated on a HYPERSIL column (250×4.5 mm 5μ Hypersil Sax). Five millimolar $NH_4H_2PO_4$, pH 3.3, was used as mobile phase. The elution was carried out at a flow rate of 1.5 mL/min. Finally, 6-MP and its metabolites were separated utilizing the same procedure as described above in HPLC system for HGPRT assay.

Total RNA extraction, first-strand cDNA synthesis, and RT-PCR. About 25 million cells of each cell line were centrifuged and collected as pellets, and RNA extraction was done according to manufacturer's instructions (RNeasy Midi Handbook; Qiagen, KEBO Laboratory, Spånga, Sweden). The concentrations and purities of RNAs were measured by employing a RNA/DNA calculator (GeneQuant, Pharmacia Biotech, Cambridge, UK). About 5 μg of total RNA was used for cDNA synthesis using an RNA PCR kit, according to manufacturers' instructions (GeneAmp; Perkin-Elmer).

Real-time quantitative PCR. The cDNA samples were used as templates whereas an endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified as a positive control and used for normalization of the different template values. To amplify the cDNA, 100 ng of the reversed transcribed cDNA from each cell line was subjected to 40 cycles of real-time quantitative-PCR in 20 μl of total reaction volume. CNT1-3, ENT1-2, MRP4-5, and GAPDH probes and primers were used to flank the target DNA sequence. Each real-time TaqMan PCR (20 μl) consisted of the cDNA template (except for the negative controls) and H_2O to a final volume of 9 μl, 1 μl of TaqMan® Gene Expression Assay in 20× format (containing the target primers and TaqMan probe), and 10 μl of TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG (according to manufacturer's instruction "TaqMan® Gene Expression Assays," Applied Biosystems). Aliquots were then amplified by an initial period of 2 min at 50 °C and 10 min at 95 °C followed by 40 concurrent cycles involving denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The samples were placed in 96-well optical PCR plate (N 801-0560, Perkin-Elmer) and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). For relative quantification of mRNAs, the following arithmetic formula was used according to the Perkin-Elmer Instruction Manual of 1997: Relative alteration $= 2^{-\Delta\Delta CT,}$ where CT is the point (cycle) at which the amplification plot crosses the threshold and ΔΔCT represents (CT of target–CT of GAPDH) of each resistant cell line—(CT of target–CT of GAPDH) of MOLT4/WT cells. Then the mRNA gene expressions of each gene in resistant sublines were presented as percentage of gene expression in MOLT4/WT cells after normalization against GAPDH (Fig. 4).

siRNA transfection and labeling. One day prior to transfection, the cell cultures were split and the sub-cultures grown in the usual medium. On the day of transfection, the cells were washed in Opti-MEM (Life Technologies Inc., Rockville, MD) and 2×10^6 cells were then resuspended in 400 μL medium for incubation with 1 μg of siRNA of target gene or non-specific siRNA for 10 min in a 0.4 cm electroporation cuvette at room temperature. This mixture was pulsed once (voltage: 340 V, capacitance: 1 mF, Resistance: ∞, and Cuvette: 4 mm. TC: 38 ms) using an electroporator (Gene Pulser Xcell Electroporation system, Bio-Rad) and thereafter incubated at room temperature for 30 min and transferred to 96-well plates (Becton–Dickinson, Franklin Lakes, NJ).

In one experiment, in order to visualize their association with nucleus, the duplex siRNAs were labeled before electroporation, with a red chromophore Cy3 using the siRNA Labelling Kit according to the manufacturer's instructions (Ambion, Huntingdon, Cambridgeshire, UK). The MTT and transport assays on siRNA treated cells targeting CNT3, ENT1, and ENT2 were performed 72 h after transfection, and the cells that had been transfected with non-specific siRNA were used as controls. All steps were done as explained in "Assaying cytotoxicity" and "Transport assay."

Effects of Na^+ ions and inhibitors of ENT1 on 6-MP uptake. When investigating the influence of Na^+, NBTI, and dipyridamole on 6-MP transport, and in order to minimize the interference of intracellular metabolism, the transport assays were performed within 1 min. Following collection by centrifugation, the cells were resuspended in Waymouth buffer (135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.8 mM $MgSO_4$, 28 mM glucose, and 13 mM Hepes, pH 7.4) at a density of 1–10^6/mL. For the Na^+-free uptake assay, the NaCl in this buffer was replaced by LiCl. Uptake was initiated by addition of [^{14}C]6-MP to the final concentration of 10 μM, at 37 °C. Termination of process and detection of intracellular radioactivities were performed as explained in "Transport assay."

Statistical analysis. The data were analyzed with Student's *t* test and a *p* value <0.05 considered to be statistically significant. The calculations were performed with the Prism version 3.0a for Macintosh (GraphPad Software, San Diego, CA).

Results

Selection and characterization of 6-MP- and 6-TG-resistant cell lines

In order to assess the mechanism underlying resistance to thiopurines, we selected two resistant cell lines by exposing the parental MOLT4 cells during 12–18 cycles (72 h each) to stepwise increasing concentrations of 6-MP or 6-TG (from 0.05 to 5 μM). The resulting cell lines were similar to the parental cells with respect to growth rate and various cell cycle parameters (data not shown). Characterization of employing a 72-h exposure to the drugs revealed that the cells selected for resistance to 6-MP (MOLT4/MP) were more resistant to both 6-MP and 6-TG ($IC_{50}S = 23.5 \pm 16$ and 18.3 ± 4.6 μM, respectively) than wild-type MOLT4 cells ($IC_{50}S = 3.3 \pm 0.9$ and 2.1 ± 0.4 μM) (Fig. 2). Respectively, the cell line selected for resistance to 6-TG (MOLT4/TG) was more resistant to both 6-TG and 6-MP ($IC_{50}S = 48 \pm 13$ and 63 ± 21 μM) (Fig. 2). Both resistant cell lines displayed collateral sensitivity to growth inhibition by the methylated analogue of 6-MP, methyl mercaptopurine riboside (meMPR). The IC_{50} values for MOLT4/MP and MOLT4/TG cells were 0.2 ± 0.01 and 0.41 ± 0.24 μM, respectively, and that of MOLT4/WT was 1.4 ± 0.4 μM. Resistant cells were stable in at least 10 passages without addition of drugs to the culture.

The enzymatic activities of HGPRT, TPMT, and IMPDH in resistant cells

HGPRT is the key enzyme in connection with the activation of 6-MP and 6-TG. Reduction or absence of this activity has previously been proposed as a mechanism for resistance to thiopurines [4]. In crude extracts of the parental MOLT4 cells, this activity was 643 ± 65 pmol/min/mg proteins and there was no significant decrease in resistant cells. Likewise, with respect to the methylating activity of TPMT and the activity of IMPDH (the second enzyme involved in phosphorylation of 6-MP), there was no difference between these three cell lines (control values for TPMT was 4.9 ± 1.0 and for IMPDH was 28.0 ± 2.6 pmol/min/mg proteins).

Transport of 6-MP

Availability of radiolabelled 6-MP (Moravek Biochemicals, CA) allowed us to investigate the initial transport of [^{14}C]6-MP across the cell membrane. At 60th min the uptake by the parental MOLT4 cells, 6-MP- and 6-TG-resistant sublines was 18 ± 1, 4.3 ± 0.6, and 2.9 ± 0.5 nmol/10^7 cells, respectively (Fig. 3). Following 1 min exposure to 10 μM [^{14}C]6-MP, the cellular concentrations in parental, 6-MP-resistant, and 6-TG-resistant cells were 7.1 ± 1.1, 2.2 ± 0.4, and 1.8 ± 0.4 nmol/10^7 cells, respectively (Fig. 3, inset). Analysis of intracellular metabolites of 6-MP over 60 min employing HPLC revealed that corresponding ratios of amounts of these metabolites among wild-type and resistant cell lines were almost the same. We found $79 \pm 11\%$ of intracellular 6-MP as TIMP plus thioxanthosine monophosphate plus thioguanosine monophosphate and $15 \pm 8\%$ as methylated forms of these three metabolites (methylthioinosine monophosphate, meth-

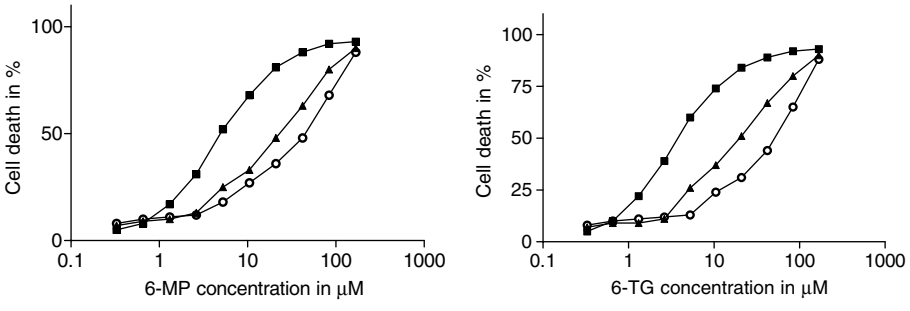

Fig. 2. Resistance to 6-MP and 6-TG in MOLT4/WT (■), MOLT4/6-MP (▲), and MOLT4/6-TG (○) cells. Cell growth was assessed using MTT proliferation assay. The cells were incubated with a range of concentrations of 6-MP or 6-TG for a period of 72 h under a humidified atmosphere containing 5% CO_2 at 37 °C. Then cell viabilities were determined and expressed as percentage of the viable control (nondrug treated) cells as described in Materials and methods. Data represent means ± SD of three separate experiments.

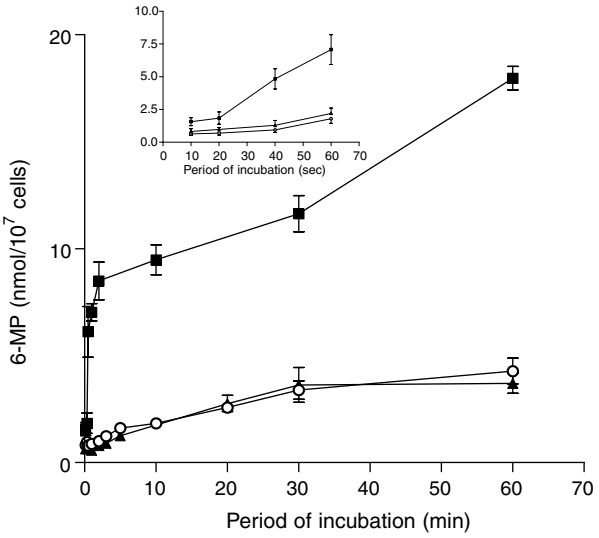

Fig. 3. Accumulation and the initial transport (inset) of [^{14}C]6-MP in MOLT4/WT (■), MOLT4/6-MP (▲), and MOLT4/6-TG (○) cells as a function of time. The concentration of [^{14}C]6-MP in the medium was 10 μM. Initial transport of [^{14}C]6-MP, 10, 20, 30, and 60 s incubation. 6-MP was separated and measured by HPLC as explained in "HPLC system." The absolute values for the wild-type and 6-MP- and 6-TG-resistant cell lines after 1 min were 7.1 ± 1.1, 2.2 ± 0.4, and 1.8 ± 0.4 nmol/10^7 cells, respectively. After 60 min of incubation, the overall uptake of 6-MP by the parental MOLT4 cells and 6-MP- and 6-TG-resistant sublines detected by HPLC using liquid scintillation was 18 ± 1, 4.3 ± 0.6, and 2.9 ± 0.5 nmol/10^7 cells, respectively. The results presented in this figure are means ± SD of three independent experiments.

ylthioxanthosine monophosphate, and methylthioguanosine monophosphate).

Real-time quantitative PCR

In attempt to understand the molecular basis of the transport defect, we then determined the levels of mRNA expression of MRP4, MRP5, and the suspected nucleoside transporter genes (NTs), using real-time quantitative PCR with GAPDH as internal standard. The levels of mRNA expression of CNT3 in MOLT4/MP and MOLT4/TG cells showed 42% and 77% reduction, in comparison with wild-type cells (Fig. 4A). The level of mRNA expression of ENT2 was also decreased in both MOLT4/MP and MOLT4/TG cells by 70% and 80%, respectively (Fig. 4B). No significant alterations of gene expression of ENT1, MRP4, and MRP5 were detected, and mRNA gene expression levels of CNT1 and CNT2 were similar (data not shown).

Silencing of CNT3, ENT1, and ENT2 genes in MOLT4 cells

Regarding the reduced expressions of CNT3 and ENT2 mRNA among 6-MP- and 6-TG-resistant cells, and in order to evaluate further the probable role of altered nucleoside transporter-mediated uptake of 6-MP in the resistance of MOLT4/MP and MOLT4/TG cells, we used siRNA gene silencing against CNT3 and ENT2 as well as ENT1 genes in MOLT4 wild-type cells. To ensure that this approach would silence even target genes carrying mutations, we employed pools of several siRNAs directed towards distinct sites in the target genes. The efficiency of transfection of MOLT4 cells was visualized by labeling the siRNAs with the red fluorochrome cy3 using a silencer siRNA Labelling Kit (Fig. 5A). Electroporation of MOLT4/WT cells with CNT3-, ENT2- (Fig. 5B), and ENT1-siRNA (data not shown) significantly decreased the gene expressions at the mRNA level 24 h after transfection. We detected approximately $47 \pm 13\%$ reduction in 6-MP transport 72 h after transfection with CNT3-siRNA and in comparison to the MOLT4 cells transfected with non-specific siRNA (Fig. 5C). Uptake of 6-MP was reduced by $21 \pm 4\%$ in the cells treated with ENT2-siRNA (Fig. 5C), but not significantly in the cells which had been transfected by ENT1-siRNA (data not shown).

Following siRNA transfection, and after 3 days, the cells were examined for tolerance to 6-MP and 6-TG, employing MTT assay. After transfection with siRNA targeting CNT3 , the MOLT4 wild-type cells tolerated higher concentrations of 6-MP, compared to MOLT4 cells which had been transfected with non-specific siRNA

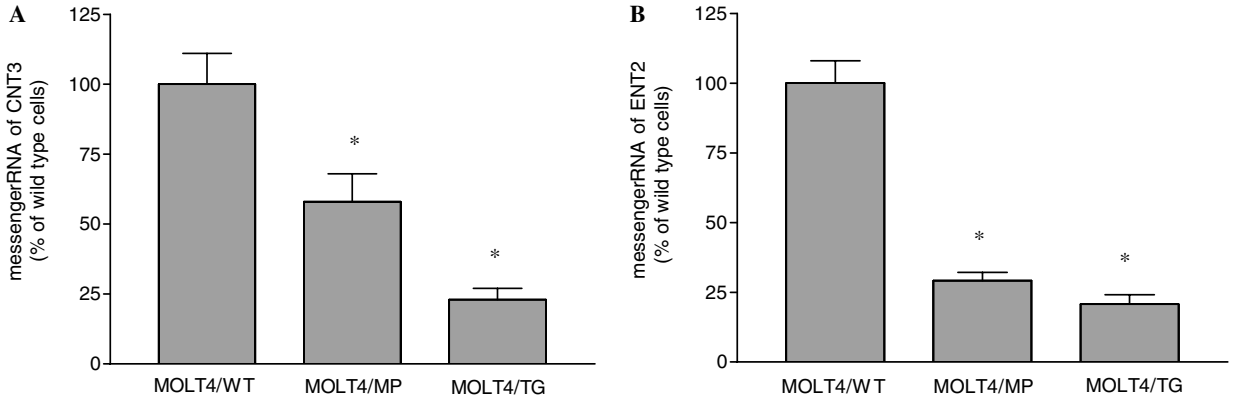

Fig. 4. Relatively low expression of CNT3 (A) and ENT2 (B) in 6-MP and 6-TG cells, as determined by using real-time quantitative PCR. Quantitation of mRNAs was performed as described in the Materials and methods. As an internal control, GAPDH was quantified as a positive control and used for normalization of the different template values. $^{*}P < 0.05$.

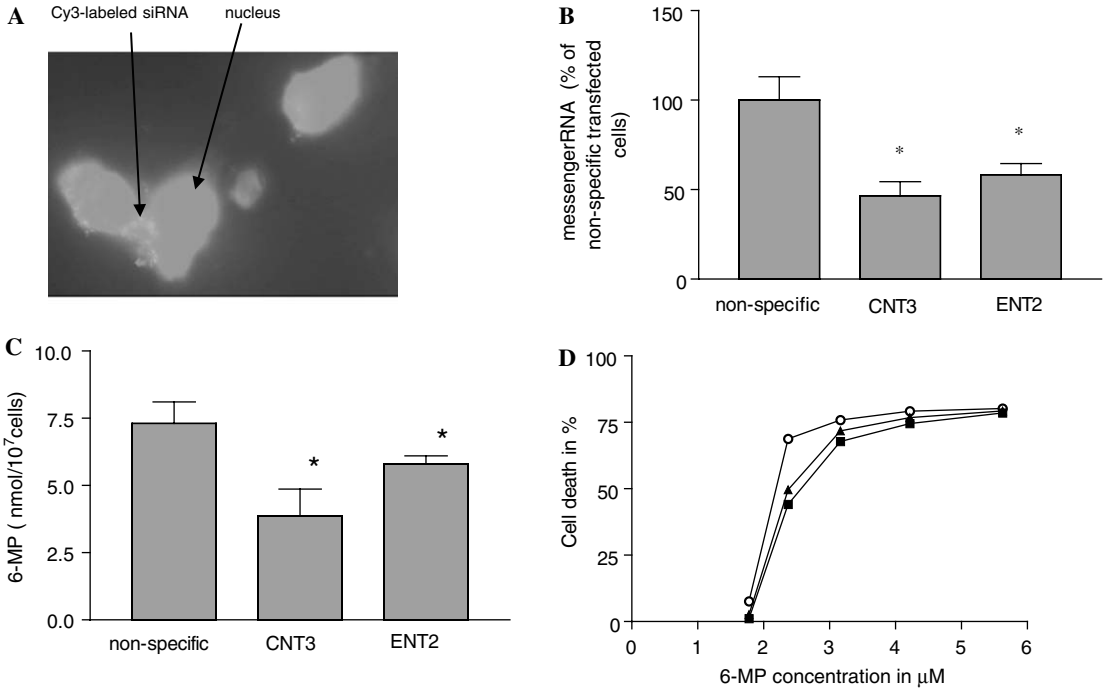

Fig. 5. (A) Visualization of MOLT4/WT cells transfected with CNT3-siRNA. The cells were mounted on microscope slides using VectaShield (Vector Laboratories, Inc., Burlingame, CA) with DAPI (4′,6-diamidino-2-phenylindole) and thereafter examined employing the appropriate fluorescence filters. The siRNA (light) can be seen to be associated with the nucleus (gray) of a cell. (B) The levels of CNT3 and ENT2 mRNAs in the cells transfected with siRNA targeting these genes, compared to those of the cells transfected with siRNA of random sequence, as determined by real-time quantitative RT-PCR analysis. (C) Decreased transport of [^{14}C]6-MP into the cells transfected with CNT3- or ENT2-specific siRNA following a 1 min exposure to 10 μM of the drug. The values of accumulation for the non-specific-, CNT3-, and ENT2-transfected cells were 7.3 ± 0.8, 3.87 ± 0.1, and 5.82 ± 0.3 nmol 6-MP/10^7 cells. (D) MTT proliferation assay showing that cells are more sensitive to 6-MP when they have been already transfected with non-specific siRNA (○), compared to when they have been transfected with siRNA targeting CNT3 (■) or ENT2 (▲). The MOLT/WT cells were incubated with a range of concentrations of 6-MP for a period of 72 h under a humidified atmosphere containing 5% CO_2 at 37 °C. Then cell viabilities were determined and expressed as percentage of the viable control (nondrug treated) cells as described in Materials and methods. $^*P < 0.05$.

($IC_{50}S = 2.7 \pm 0.3$ and 2.1 ± 0.2 μM, respectively) (Fig. 5D). The corresponding IC_{50} values for the MOLT4 cells transfected with ENT2-siRNA were 2.5 ± 0.4 μM, and in the case of the MOLT4 cells after transfection with ENT1 were 2.2 ± 0.6 μM.

Effects of Na^+ ions and inhibitors of ENT1 on 6-MP uptake

In order to further confirm the role of Na^+-dependent CNT3 in cellular uptake of 6-MP to MOLT4 cells, and ruling out the interference of ENT1 in this uptake, the effects of sodium ions, and of two classic ENT1-inhibitors, NBTI and dipyridamole, were characterized. Transport of 6-MP (10 μM) in 1 min was found to be Na^+-dependent, being reduced in the absence of this ion by $55 \pm 2\%$ (Fig. 6). On the other hand, transport of 6-MP was not altered in the presence of up to 100 nM NBTI or dipyridamole (data not shown).

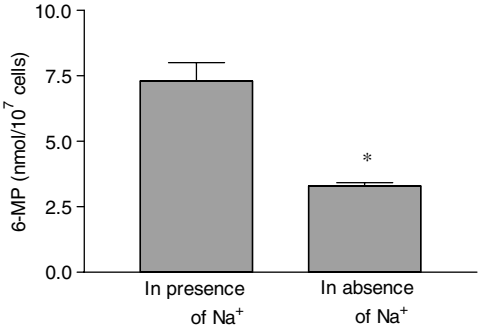

Fig. 6. Comparison of the initial transport of [^{14}C]6-MP into MOLT4/WT cells in the presence and absence of sodium ions, i.e., in Waymouth buffer containing 135 mM NaCl or 135 mM LiCl, respectively. The incubation time was 1 min and the temperature 37 °C. The results are presented as means ± SD of at least three independent experiments. $^*p < 0.05$.

Discussion

Although the thiopurine analogs, 6-MP and 6-TG, are widely used in cancer chemotherapy, the mechanisms of their action and the mechanisms by which cells become resistant to these drugs remain to be elucidated in detail. One of the most extensively characterized mechanisms for in vitro resistance to 6-MP and 6-TG is the absence of HGPRT activity, which transfers a phosphoribosyl moiety to these molecules in the first step of their intracellular metabolism (Fig. 1). In a previous clinical study, low HGPRT activity was demonstrated to be correlated with a poorer prognosis in precursor B-ALL patients [18]. How-

ever, there is currently no evidence supporting a significant contribution of this mechanism to clinical resistance to thiopurines, probably as very low level of HGPRT is sufficient to generate the cytotoxic nucleotides [18].

Alteration in TPMT activity has also been shown to influence the sensitivity of cells to the cytotoxic effects of both 6-MP and 6-TG, but in opposite directions [22]. A decrease in the activity of this methylating enzyme could result in the production of lower levels of meTIMP following exposure to 6-MP, thus conferring resistance to this drug. On the other hand, such a decrease could increase the sensitivity of cells to 6-TG by leading to lower levels of meTGMP and, consequently, increase the levels of DNA-thioguanine nucleotides (DNA-TGNs) [5]. Neither of our resistant cell lines demonstrated any change in the level of either TPMT activity in cell extracts or TPMT mRNA, nor did they exhibit any significant alteration in the activity of IMPDH, the second enzyme involved in the metabolic conversion of 6-MP to TGNs. Similar levels of HGPRT and TPMT enzyme activities in all our cell lines ruled out the most characterized metabolisms of resistance to thiopurines.

Although there are several studies that explain degrees of resistance to nucleoside analogs as a consequential effect of defective nucleoside transporter-mediated uptake of nucleoside analogs [9–13], there is no report that explains an acquired resistance to thiopurines as a result of defective transport. However, down-regulation of CNT3 and ENT2 gene expressions which was detected by quantitative real-time PCR was correlated with resistance to 6-MP and 6-TG in both resistant cells. In a recently published paper, Zaza et al. [23] reported a 33–45% reduction of TGN in cells of ALL patients upon 24 h co-exposure to 6-MP and NBTI. However in the case of our MOLT4/WT cells, exposure to 100 nM concentration of NBTI and dipyridamole could not significantly inhibit the initial transport of 6-MP. Besides, transfecting the MOLT4 cells with siRNA targeting the ENT1-mRNA did not influence the transport of 6-MP significantly (Fig. 5C).

Even though there is at present no convincing evidence that MRP efflux proteins may play a role in the development of resistance against anti-malignancy drugs in vivo, several in vitro studies do suggest that a certain degree of resistance is associated with enhanced activities of certain of these proteins [24,25]. The ability of these efflux proteins to transport 6-MP and 6-TG out of the cell, as well as resistance to these drugs in cells that were transfected with the gene for MRP4 or MRP5, has been described [26]. However, in the present investigation there were no differences in the levels of expression of mRNAs encoding the efflux proteins in our cell lines using real-time PCR, thereby ruling out this mechanism of resistance.

Briefly, in both of our sublines of leukemia MOLT4 cells selected for resistance to 6-MP and 6-TG, defective cellular uptake could account for resistance, which is a mechanism that has not been reported previously. The evidence for this conclusion is as follows: (1) The initial transport of 6-MP in our resistant cell lines was significantly reduced (Fig. 3). (2) Analysis of functionally characterized NTs by real-time quantitative PCR revealed a selective decrease in the expression of CNT3 and ENT2 mRNAs in both resistant cell lines (Figs. 4A and B). (3) Reduction of the expression of CNT3 and ENT2 in wild-type MOLT4 cells by siRNAs (Fig. 5B) was accompanied by a marked decrease in their uptake of 6-MP (Fig. 5C), and increase in tolerance to 6-MP toxicity (Fig. 5D), emphasizing the role played by CNT3 and ENT2 in 6-MP uptake. (4) High concentrations of NBTI and dipyridamole did not influence transport of 6-MP by wild-type MOLT4 cells, ruling out any significant involvement of ENT1 in this process. (5) The transport of 6-MP by wild-type MOLT4 cells was significantly reduced in the absence of Na^+ ions, further verifying the involvement of a concentrative NT in this transport (Fig. 6).

Interestingly, cytotoxicity assays have revealed that meMPR, a methylated metabolite of 6-MP, can bypass resistance to the parental drug. However, in our resistant sublines, the toxicity of the methylated metabolite, meMPR, was actually elevated, an interesting finding that requires further investigations.

In conclusion, the present study provides the first evidence that impairment of transport as a result of decreased expression of CNT3 and ENT2 can on its own confer resistance to thiopurines. It seems probable that this phenomenon may be particularly prevalent after long-term exposure to 6-MP and 6-TG, thereby, in a clinical setting impairing their cytotoxic activity. It will be important to ask whether this mechanism of resistance is clinically relevant, e.g., by looking for decreased expression of nucleoside transporters in patient samples collected at the time of relapse, relative to specimens at the time of initial diagnosis. Since all NTs have been shown to play a role in the cellular uptake of anticancer nucleoside analogs, determination of the abundance of these transporters may be valuable in predicting the efficacy of such drugs in individual patients.

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