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Molecular mechanisms underlying the enhanced sensitivity of thiopurine-resistant T-lymphoblastic cell lines to methyl mercaptopurineriboside

Alan Kambiz Fotoohi [a], *Anna Wrabel* [a], *Ali Moshfegh* [a], *Curt Peterson* [b], *Freidoun Albertioni* [a,*]

[a] *Department of Oncology and Pathology, Cancer Center Karolinska (CCK), Karolinska Hospital and Institute, 171 76 Stockholm, Sweden*
[b] *Department of Medicine and Care, Faculty of Health Science, Linköping University, Linköping, Sweden*

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Abbreviations:
DNPS, *de novo* purine synthesis
ENT1, equilibrative nucleoside transporter 1
HGPRT, hypoxanthine-guanine phosphoribosyl transferase
IC_{50}, concentration of the drug that inhibits cell growth by 50%
6-MP, 6-mercaptopurine
meMPR, methylmercaptopurine riboside
meTIMP, methylthioinosine monophosphate
NT(s), human nucleoside transporter(s)

ABSTRACT

Methylmercaptopurine riboside (meMPR), a cellular metabolite of 6-mercaptopurine (6-MP), is a potent inhibitor of *de novo* purine synthesis (DNPS). Human MOLT4 T-lymphoblastic leukaemia cells that have acquired resistance to 6-MP or 6-thioguanine (6-TG) as a consequence of defective transport exhibit enhanced sensitivity to meMPR. HPLC-based analysis of the transport of meMPR revealed normal uptake of this compound by our thiopurine-resistant cell sublines, suggesting a route of transport distinct from that for 6-MP and 6-TG. Studies on the wild-type parental leukemic cells showed that adenosine, dipyridamole and nitrobenzylthioinosine inhibit uptake of meMPR to a significant extent, whereas Na^+ ions have no influence on this process. Transfection of these leukemic cells with small interference RNA molecules targeting the gene encoding the first member of the family of equiliberative nucleoside transporters (ENT1) strongly reduced the initial rate of meMPR transport. Our resistant cell lines exhibited 30–52% reductions ($p < 0.005$) in their levels of mRNA encoding several proteins involved in *de novo* purine synthesis, i.e., aminoimidazole carboxamide ribonucleotide formyltransferase, glycinamide ribonucleotide transformylase and guanine monophosphate synthetase. Consequently, the rate of *de novo* purine synthesis in these resistant sublines was decreased by 50%. Furthermore, the levels of ribonucleoside triphosphates in these cells were significantly lower than in the non-resistant parental cells.

In combination, a reduced rate of *de novo* purine synthesis together with low levels of ribonucleoside triphosphates can explain the enhanced sensitivity of our thiopurine-resistant cell lines to meMPR. In this manner, meMPR bypasses the mechanisms of resistance to thiopurines and is even more cytotoxic towards resistant than towards wild-type cells.

* *Corresponding author*. Tel.: +46 8 517 75832; fax: +46 8 517 75042.
E-mail address: Freidoun.albertioni@cck.ki.se (F. Albertioni).
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NTP, ribonucleoside triphosphate pools
6-TG, 6-thioguanine
TPMT, thiopurine methyltransferase

1. Introduction

The thiopurines 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) are cornerstones in chemotherapy of childhood acute leukaemia. Unfortunately, intrinsic and acquired resistance has become a major problem in connection with such treatment and numerous efforts are being made to find alternative drugs which can bypass this resistance. In this context, certain thiopurine analogues and their nucleosides have been shown to effectively kill some tumour cell lines and such compounds might therefore prove useful in treating patients whose disease has become resistant to 6-MP and/or 6-TG [1].

We have recently demonstrated that in the case of two separate MOLT4 cell lines that have acquired resistance to 6-MP and 6-TG, the primary mechanism of resistance involves reduced uptake of these drugs via the third member of the family of concentrative nucleoside transporters (CNT3) and the second member of the equiliberative nucleoside transporter family, ENT2 [2]. The most thoroughly characterized mechanisms of resistance to thiopurines, i.e., a lack of hypoxanthine-guanine phosphoribosyl transferase (HGPRT) or altered thiopurine methyltransferase (TPMT) activity, were not exhibited by these cell lines. Interestingly, these 6-MP- and 6-TG-resistant MOLT4 cells exhibited enhanced sensitivity to the cytotoxicity of methyl mercaptopurine riboside (meMPR), a methylated metabolite of 6-MP.

Prior investigations have revealed that the formation of meTIMP from meMPR in cultured cells results in cytotoxicity due to inhibition of *de novo* purine synthesis [3]. This phosphorylation of meMPR is catalyzed by adenosine kinase and the presence of an attenuated level of this activity is a well-established mechanism for resistance to the toxic effects of this compound [4]. In phase I and II trials MeMPR has been included in combination drug treatment of the patients suffering from advanced forms of cancer [5–7].

In light of the fact that 6-MP and MeMPR exert their cytotoxicity via a common pathway, we were interested in determining why our 6-MP- and 6-TG-resistant cell lines exhibit enhanced sensitivity to meMPR. We show here that the influx of meMPR into these cells is unaltered and, at the same time, the rate of *de novo* purine synthesis and the cellular pools of ribonucleoside triphosphate are significantly reduced, which might explain this enhanced cytotoxicity. We have also examined the route of influx of meMPR into MOLT4 cells using classic inhibitors and, moreover, extended these studies by characterizing meMPR transport in cells transfected with small interfering RNAs designed to silence the ENT1 gene.

2. Materials and methods

2.1. Chemicals

6-Mercaptopurine, 6-thioguanine, adenosine monophosphate, hypoxanthine, methylmercaptopurine riboside, nitrobenzyl thioinosine (NBTI), dipyridamole, adenine, guanine, cytidine, adenosine, guanosine, uridine and thymidine (Sigma–Aldrich, Stockholm, Sweden); [8] formic acid (58 Ci/mmol) (Amersham, Uppsala, Sweden); [2,8-^{3}H]-adenosine (48 Ci/mmol) (Moravek Biochemicals Inc., Brea, CA, USA); monoammonium phosphate ($NH_4H_2PO_4$) (Merck, Darmstadt, Germany); RPMI 1640, heat-inactivated fetal calf serum (FCS), L-glutamine, and penicillin–streptomycin (Life Technologies, Paisley, United Kingdom); TaqMan reagents and gene expression assays (including the assays for glycinamide ribonucleotide transformylase (GART) (no. Hs00531926_m1), aminoimidazole carboxamide ribonucleotide formyltransferase (ATIC) (no. Hs00269671_m1), guanine monophosphate synthetase (GMPS) (no. Hs00269500_m1), ENT1 (no. Hs00223220_m1) and gylceraldehyde-3-phosphate dehydrogenase (GAPDH) (no. Hs99999905_m1)) (Applied Biosystems, Stockholm, Sweden); and ENT1 SMARTpool siRNA duplex (proprietary target sequences) (Dharmacon Research, Inc., Lafayette, CO) were purchased from the sources indicated. meTIMP was synthesised in our laboratory.

2.2. Cell lines

The acute lymphoblastic leukemia T-lymphoblastic MOLT4 cell line (obtained from American Type Culture Collection, Rockville, MD) was subcultured twice weekly at 37 °C in RPMI-1640 medium supplemented with FCS (10%), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) under humidified air containing 5% CO_2. 6-MP- or 6-TG resistant subclones of this original parent cell line were selected for by exposure to increasing concentrations of 6-MP or 6-TG, until a final concentration of 5 μM was reached. These subclones were subsequently cultured for at least three passages in drug-free medium for at least three passages prior to experimental use during the logarithmic phase (approximately $0.8–1.5 \times 10^6$) of their growth. A Coulter Multisizer (Coulter Electronics, Luton, United Kingdom) was employed to count the cells.

2.3. Assay of cytotoxicity

Cytotoxicity assay was assayed as described by Mosmann et al. [9]. In brief, 100-μl aliquots of a suspension containing 2×10^5 cells/ml were placed in triplicate in the wells of 96-well round-bottomed microtiter plates each containing 5 ml of drug at various concentrations. Drug-free wells were employed as controls and cell-free wells as blanks. Following

incubation for 72 h under humidified air containing 5% CO_2, an aqueous solution (5 mg/ml) of tetrazolium salt (MTT) was added to each well and the plates then incubated for an additional 4 h at 37 °C. The formazan salt crystals formed were dissolved with 100 μl 10% SDS dissolved in 10 mM HCl overnight at 37 °C and thereafter the absorbance of the resulting solution at a wavelength of 540 nm (using 650 nm as the reference wavelength) was determined utilizing an ELISA plate reader (Labsystems Multiscan RC, Helsinki, Finland). IC_{50} values were defined as the drug concentrations at which cell growth was inhibited by 50% in comparison to the growth in drug-free control wells.

2.4. Transport of meMPR

Uptake of meMPR was monitored by first washing logarithmically growing cells with RPMI 1460 medium containing HEPES, pH 7.4, by centrifugation and then incubating these cells (resuspended in the same medium at a density of 2×10^6 cells/ml) with 10 μM meMPR (final concentration in the culture medium) at 37 °C for 1 min. Thereafter, the cells were pelleted by centriguation (5 min at 1800 × *g* and 4 °C) and subsequently washed twice with ice-cold PBS. The resulting cell pellets were then resuspended in 100 μl 1 M perchloric acid (PCA) and this suspension was neutralized with 25 μl 4 M KOH. After mixing, this extract was centrifuged for 10 min at 4000× *g* in order to remove cell debris and 50 μl of the protein-free supernatant thus obtained injected into an HPLC system for determination of the intracellular levels of meMPR and meTIMP. This HPLC system consisted of a CM4000 pump (Milton Roy, LDC Division, USA) and a CMA-240 auto sampler (Carneige Medicine, Stockholm, Sweden) equipped with a SPD-6A ultraviolet spectrophotometric detector (Shimadzu, Tokyo, Japan). Separation of meMPR and meTIMP was performed on a Hypersil Duet C18/SAX column. An aqueous solution of 5 mM $NH_4H_2PO_4$ containing 10% ethanol, pH 2.9, was passed through a filter with 0.22-μm pores (Millipore, Ireland) and degassed prior to use as the mobile phase. Elution was carried out at a flow rate of 2 ml/min and a wavelength of 290 nm was utilized for detection and quantitation.

2.5. De novo purine synthesis (DNPS)

The rate of DNPS was determined on the basis of the amounts of radiolabeled adenine and guanine formed during a 2-h incubation of the cells with [^{14}C]formate (at a final specific radioactivity of 50 dpm/pmol), according to the procedure described previously by Masson et al. [8]. This rate is expressed as femtomoles of radiolabelled adenine and guanine synthesized per nanomole of total intracellular adenine plus guanine during 1 h of incubation (fmol/nmol/h).

2.6. Determination of ribonucleoside triphosphate pools

For this purpose, the cells were first sub-cultured for more than two passages in RPMI medium containing L-glutamine, but without penicillin or streptomycin. Following two washes with ice-cold PBS, exponentially growing cells were counted and extracted with ice-cold perchloric acid, neutralized with 4 M potassium hydroxide and centrifuged as described above, after which an aliquot of the resulting protein-free supernatant was injected onto a Particil-10 SAX anion exchange column (250× 4 mm, Whatman). Subsequently, intracellular nucleoside triphosphate (NTP) levels were quantitated employing an HPLC procedure described previously [10], with determination of the levels of ATP, GTP and UTP on the basis of the absorbance at 260 nm and of CTP at 280 nm.

2.7. Assay of adenosine kinase activity in cell lysates

In brief, the adenosine kinase activity of crude cell extracts was assayed as follows: cells in their logarithmic phase of growth were suspended at a density of 10^6 ml^{-1} in an extraction buffer containing 50 mM Tris–Cl, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol and 0.5% Nonidet P40, with a pH of 7.6. This suspension was then frozen in liquid nitrogen and thawed three times, followed by centrifugation at 11,800 × *g* for 5 min at 4 °C to obtain a particle-free lysate. The reaction mixture contained 50 mM Mops, 100 mM KCl, 6 mM $MgCl_2$, 100 μM dithiothreitol, 50 μM β-methylene adenosine diphosphate, 200 μg bovine serum albumin, 100 μM EHNA, [^{3}H] adenosine or meMPR and 2–3 μg protein in a final volume of 25 μl. The concentrations of substrates chosen were approximately 10 times higher than the corresponding K_m values. After 30 min of incubation at 37 °C, the reaction was terminated by placing the samples on ice. Following addition of perchloric acid and subsequent neutralization with KOH (see above), the samples were centrifuged at 13,500 rpm for 5 min at 4 °C and aliquots of the supernatants thus obtained injected into the HPLC system. This HPLC system consisted of a CM4000 pump (Milton Roy, LDC Division, USA) and a CMA-240 autosampler (Carneige Medicine, Stockholm, Sweden) equipped with a 500TR flow scintillation counter (Packard, IL, USA). meMPR and meTIMP were separated employing a Hypersil Sax ion-exchange column (250× 4.5 mm) and a mobile phase consisting of 5 mM $NH_4H_2PO_4$ containing 10% methanol (v/v), with a pH of 3.3. Elution was carried out at a flow rate of 1.5 ml/min and the wavelength of detection set at 290 nm. Adenosine and adenosine monophosphate were separated on a HICROM anion exchange column (250× 4 mm, Whatman) employing 0.4 M $NH_4H_2PO_4$, pH 3.3, as the mobile phase and elution at a flow rate of 2 ml/min. Adenosine kinase activity is expressed as pmol of adenosine monophosphate or meTIMP formed per minute and mg of crude cellular protein.

2.8. Protein determination

The protein concentrations of crude cell extracts were determined according to the procedure developed by Lowry et al. (Lowry 1952# 50) using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

2.9. Extraction of total RNA, synthesis of first-strand cDNA and real-time quantitative PCR

Employing approximately 25 × 10^6 cells collected by centrifugation, total RNA was extracted by the RNeasy procedure (RNeasy Midi Handbook; Qiagen, KEBO Lab, Spånga, Sweden) in accordance with the manufacturer's instructions. The

concentrations and purities of the RNA preparations thus obtained were determined utilizing an RNA/DNA calculator (GeneQuant, Pharmacia Biotech, Cambridge, UK). About 5 μg of this total RNA was used for cDNA synthesis using an RNA PCR kit (GeneAmp; Perkin-Elmer) in accordance with the manufacturer's instructions. These cDNA samples were subsequently employed as templates together with random hexamers as the primers. The level of cDNA for gylceraldehyde-3-phosphate dehydrogenase (GAPDH), an endogenous housekeeping protein, was determined as an internal positive control and used for normalization of the levels of the other cDNAs. For amplification, 100 ng of the cDNA from each cell line was subjected to 40 cycles of real-time quantitative PCR in a total reaction volume of 20 μl. Probes and primers specific for ATIC, GART, GMPS and GAPDH were used to flank the target DNA sequences. Each real-time TaqMan PCR reaction contained the cDNA template (not added in the case of the negative controls) dissolved in 9 μl H_2O; 1 μl of TaqMan® Gene Expression Assay (containing the target primers and TaqMan probe) concentrated 20-fold; and 10 μl of TaqMan Fast Universal PCR Master Mix concentrated 2-fold (No AmpErase UNG, TaqMan® Gene Expression Assays, Applied Biosystems), in accordance with the manufacturer's instructions. These samples were initially amplified for 2 min at 50 °C and 10 min at 95 °C, followed by 40 consecutive cycles involving denaturation at 95 °C for 15 s and annealing/extension at 60 °C for1 min. This amplification was performed on 96-well optical PCR plates (N801-0560, Perkin-Elmer) in an automated fluorimeter (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). For quantitation of the relative levels of ATIC, GART and GMPS mRNAs, the following equation provided by the Perkin-Elmer Instruction Manual of 1997 was utilized: relative change = $2 - \Delta CT$, where CT is the point (cycle) at which the amplification plot crosses the threshold and ΔCT = (CT of the target mRNA − CT of GAPDH mRNA) for each resistant cell line. The level of mRNA for ATIC, GART or GMPS expressed by each resistant subline is expressed as a percentage of the corresponding level in the parent MOLT4/WT cell line, following normalization with respect to the level of GAPDH mRNA (Fig. 5).

2.10. Effects of Na^+ ions and inhibitors of ENT1 on meMPR transport

In order to characterize the role played by Na^+ ions in connection with meMPR transport, exponentially growing cells were first washed with PBS and thereafter 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) or in this same buffer with replacement of NaCl by LiCl (for assay of Na^+-free uptake) at a density of 10^7 cells/ml. For investigation of the ability of dipyridamole and NBTI, classic inhibitors of ENT1, to suppress the transmembrane uptake of meMPR, the cells were exposed to 10 nM concentrations of these inhibitors both 20 min prior to, as well as during performance of the transport assay. Uptake at 37 °C was initiated by addition of 10 μM meMPR (final concentration) and termination of uptake and detection of intracellular radioactivity was carried out as described above.

2.11. Transfection with siRNA designed to silence expression of the ENT1 gene

One day prior to transfection, the cell cultures were split and the sub-cultures grown in the routine medium. On the day of transfection, the cells were washed with Opti-MEM (Life Technologies Inc., Rockville, MD) and 2×10^6 cells then resuspended in 400 μl medium together with 1 μg siRNA targeting the ENT1 gene or of non-specific siRNA for 10 min at room temperature in a 0.4-cm electroporation cuvette. This mixture was pulsed once (with a voltage of 340 V, capacitance of 1 mF, resistance and cuvette width of 4 mm) for 38 ms using an electroporator (Gene Pulser Xcell Electroporation System, Bio-Rad) and, following incubation at room temperature for 30 min, subsequently transferred to 96-well plates (Becton Dickinson, Franklin Lakes, NJ). Seventy-two hours after transfection, the transport assays on cells transfected with siRNA designed to bind to the ENT1 gene were performed as described above, with cells that had been transfected with non-specific siRNA serving as controls.

2.12. Statistical analysis

The data were analyzed employing Student's *t*-test, with a *p*-value of <0.05 being considered statistically significant. All calculations were performed with version 3.0a of Prism for Macintosh (GraphPad Software, San Diego, CA).

3. Results

3.1. Enhanced sensitivity of 6-MP- and 6-TG-resistant cell lines to the toxic effects of meMPR

The 6-MP- and 6-TG-resistant cell lines employed here have been described in detail elsewhere [2]. The levels of expression of mRNA encoding the nucleoside transporters CNT3 and ENT2 is low in these cell lines compared to the wild-type cells; whereas the specific activities of HGPRT, IMPDH and TPMT are similar to those of the parent cells. Accumulation of 6-MP and 6-TG nucleotides by both of these resistant cell lines is much lower than by the parental MOLT4/WT cells. Analysis of growth and survival analysis revealed that MOLT4/MP cells are >5-fold more resistant to 6-MP and the MOLT4/TG are >14-fold more resistant to 6-TG. At the same time, enhanced sensitivity to meMPR was observed, with IC_{50} values of 1.4 ± 0.4, 0.2 ± 0.01 and 0.41 ± 0.24 μM for the wild-type, 6-MP-resistant and 6-TG-resistant cell lines, respectively (Fig. 1).

3.2. Transport of meMPR and adenosine kinase activity

In attempt to explain the enhanced sensitivity of the resistant cell sublines to meMPR, the rates of transport of meMPR were monitored with an HPLC procedure. Upon incubation with various concentrations of this compound, no significant difference in the accumulation of meMPR and/or meTIMP by the resistant and wild- type cells was observed (data not shown). In the case of the wild-type MOLT4 cells, the initial rate of uptake after 1 min of incubation was 22 ± 2.7 pmol/10^6 cells. It is well established that a reduction in the activity of

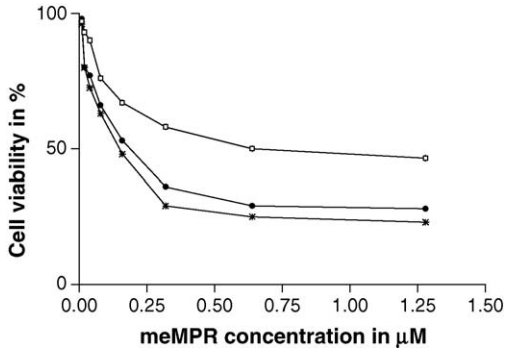

Fig. 1 – Inhibition of the growth of wild-type (MOLT4/WT, □), 6-MP-resistant (MOLT4/MP, *) and 6-TG-resistant (MOLT4/TG, ●) cell lines by meMPR. The cells were incubated with different concentrations of meMPR for 72 h under humidified air containing 5% CO_2 and at 37 °C. Subsequently, cell survival was calculated as the percentage of the cells that remained viable (as determined by the MTT assay; see Section 2) in comparison to untreated cells. The values presented are the means ± S.D. for three independent experiments.

adenosine kinase can result in less intracelluar accumulation of meTIMP, the cytotoxic phosphorylated form of meMPR, thereby yielding different degrees of resistance to meMPR [4]. Consequently, we determined the specific activity of adenosine kinase in crude extracts of all three of our cell lines with both adenosine or meMPR as substrate. The adenosine kinase activity in extracts of wild-type MOLT4 cells with adenosine as substrate was 49 ± 1.2 pmol/mg/min, while the corresponding value with meMPR was 62 ± 4 pmol/mg/min. There was no statistically significant difference between these activities in the wild-type and resistant cell lines.

3.3. *Low levels of ribonucleoside triphosphates and decreased DNPS in resistant cells*

In attempt to elucidate the biochemical basis for the enhanced sensitivity of 6-MP- and 6-TG-resistant cells to meMPR, we measured their pools of ribonucleoside triphosphates and rates of DNPS. The ribonucleoside triphosphate pools were markedly reduced in both of these sublines in comparison to wild-type MOLT4 cells (Fig. 2A). Moreover, intracellular formation of adenine and guanine from formic acid by these cells during a 2-h period, an indicator of the rate of DNPS, was attenuated by approximately 50%. The rates exhibited by wild-type, 6-MP- and 6-TG-resistant MOLT4 cells were 314 ± 33, 158 ± 14 and 62 ± 9.5 fmol/nmol/h, respectively (Fig. 2B).

3.4. *Down-regulation of the levels of mRNA coding for proteins involved in DNPS*

As assessed employing real-time quantitative PCR with GAPDH as an internal standard, the levels of ATIC, GART and GMPS mRNAs were significantly reduced in both resistant cell lines. In the case of MOLT4 cells with acquired resistance to 6-MP or 6-TG, respectively, these decreases were approximately 34 and 48% for ATIC mRNA, 48 and 52% for GART mRNA and 30 and 37% for GMPS mRNA, in comparison to the wild-type parent cells (Fig. 3).

3.5. *Effects of Na^+ ions and inhibitors of ENT1 on meMPR transport*

Since meMPR is a structural analogue of adenosine, we investigated the role of equiliberative and concentrative nucleoside transporters in uptake of this compound by wild-type MOLT4 cells. The initial rate of this uptake during a 1-min period was not significantly different in the presence and absence of Na^+ ions (data not shown), a finding that rules out any involvement of CNTs in the transport of meMPR into these cells. In contrast, nanomolar concentrations of NBTI and dipyridamole, classic inhibitors of ENT1, reduced the rate of uptake of meMPR into wild-type MOLT4 cells significantly (>30%) (Fig. 4), indicating the involvement of this transporter. Furthermore, in the presence of 1 mM adenosine the initial uptake of meMPR was strongly inhibited (>85%, $p < 0.05$) and uridine and thymidine also exerted significant inhibitory effects (>40%, $p < 0.05$). On the other hand, as expected, the

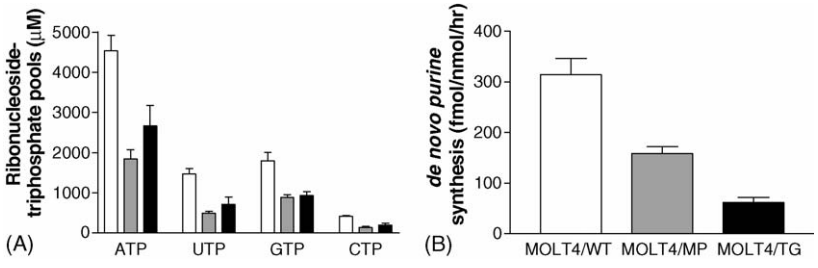

Fig. 2 – Intracellular pools of ribonucleotides and *de novo* purine biosynthesis (DNPS) in 6-MP- (MOLT4/MP) and 6-TG-(MOLT4/TG) resistant MOLT4 sublines. (A) When the ribonucleotides present in MOLT4/WT, MOLT4/MP and MOLT4/TG cells were extracted and analyzed employing an HPLC system, these pools were found to be markedly reduced in both resistant sublines in comparison to the wild-type cells. The concentrations of ATP, UTP, GTP and CTP, respectively, were 3660 ± 123, 2480 ± 142, 1690 ± 155 and 945 ± 75 μM in the case of MOLT4/WT cells; 2430 ± 189, 2120 ± 106, 1060 ± 304 and 732 ± 146 μM in MOLT4/MP cells; and 2250 ± 583, 599 ± 151, 907 ± 35 and 166 ± 44 μM in MOLT4/TG cells. (B) The rates of DNPS in MOLT4/WT and the resistant sublines were determined by quantitating the levels of radiolabeled adenine and guanine following 2 h of incubation with [^{14}C]formic acid. These values are expressed as femtomoles of newly synthesized adenine and guanine per nanomole of total intracellular adenine plus guanine per hour of incubation (fmol/nmol/h).

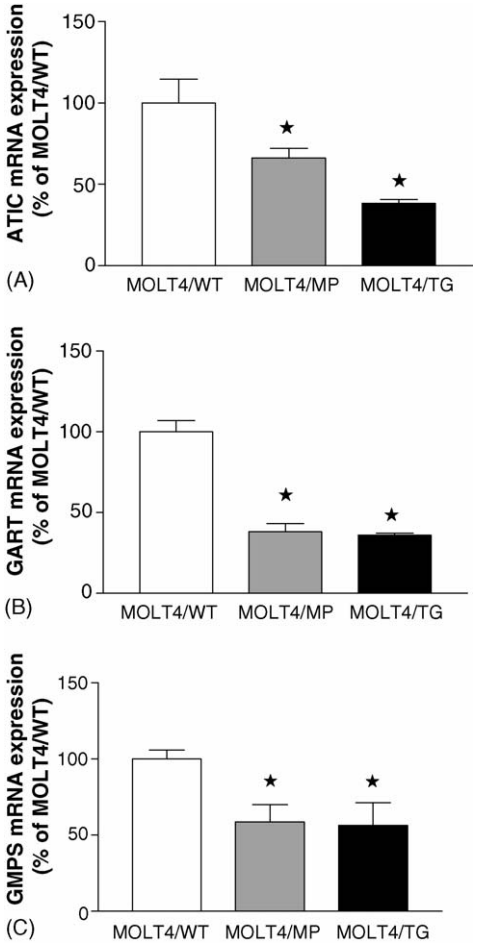

Fig. 3 – (A–C) Determination of the levels of ATIC and GART mRNA in MOLT4/WT (white), MOLT4/MP (gray) and MOLT4/TG (black) cells employing real-time quantitative PCR. The data are presented as the percentage of mRNA expressed by the resistant cells relative to the corresponding level in wild-type MOLT4 cells. These percentages for ATIC mRNA in MOLT4/MP and MOLT4/TG cells, respectively, were 66.2 ± 5.2 and 52.8 ± 13% and the corresponding values for GART mRNA were 62.2 ± 2.4 and 57.9 ± 21.6%. The values presented are the means ± S.D. of three independent experiments.

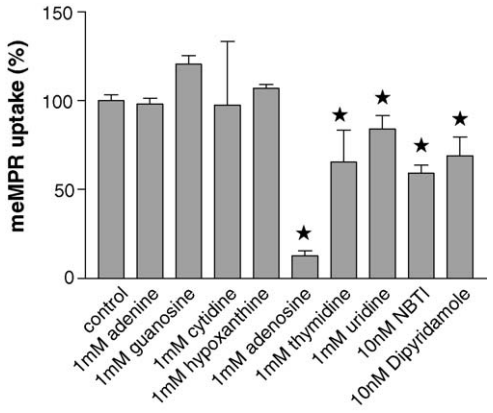

Fig. 4 – Effects of classic inhibitors of nucleoside transporters (NBTI and dipyridamole), nucleobases and nucleosides on meMPR uptake into wild-type MOLT4 cells. Initial uptake during a 1-min period was measured and expressed as a percentage of the corresponding value for untreated cells. Each data point represents the mean ± S.D. of triplicate values obtained in one representative experiment and in all cases at least two independent experiments giving similar results were performed. $^{*}p < 0.05$. The relative rates of uptake obtained under the different conditions used were as follows: control, 80 ± 3 (100%); in the presence of adenine, 79 ± 3; adenosine, 10 ± 2; guanosine, 96 ± 4; cytidine, 78 ± 29; thymidine, 52 ± 14; hypoxanthine, 86 ± 2; uridine, 67 ± 14; NBTI, 66 ± 6; and dipyridamole, 69 ± 11.

nucleobases adenine and hypoxanthine did not influence this uptake to any significant extent.

3.6. *Silencing the ENT1 gene in MOLT4 cells*

In order to evaluate the role played by the ENT1 protein in the uptake of meMPR further, the siRNA gene silencing technique was applied to wild-type MOLT4 cells. Seventy-two hours after electroporation of several siRNAs directed towards distinct sites in the target ENT1 gene into these cell, the level of ENT1 mRNA was significantly decreased (50 ± 4%), as demonstrated by real-time quantitative PCR (Fig. 5A). Moreover, the initial rate of uptake of meMPR into these cells at this same time-point was 27 ± 11% less than in the case of MOLT4 cells transfected with non-specific siRNA (Fig. 5B).

4. Discussion

Resistance to thiopurines remains a major impediment in connection with clinical administration of these drugs to cancer patients and the search for alternative drugs that can bypass the mechanisms of resistance to thiopurines continues. Of interest in this context is the observation that our two sublines of MOLT4 cells with acquired resistance against 6-MP or 6-TG exhibit enhanced sensitivity to meMPR (Fig. 1). Numerous reports describe the cytotoxicity that results from inhibition of the *de novo* biosynthesis of purines. For example, Inaba et al. [11] reported that two types of leukaemia cell lines resistant to 6-MP exhibit enhanced sensitivity to the effects of two antagonists of inhibition of DNPS. This phenomenon could be explained primarily on the basis of a defect in guanine metabolism in the resistant cells, rendering them highly dependent on production of this compound via DNPS and, thus, to the cytotoxicity of substances that inhibit this latter process. Furthermore, Li et al. [12] have recently described an inverse correlation between the level of methylthioadenosine phosphorylase, a key enzyme in DNPS, and sensitivity to the toxic effects of meMPR. These investigators found that sarcoma cells deficient in this

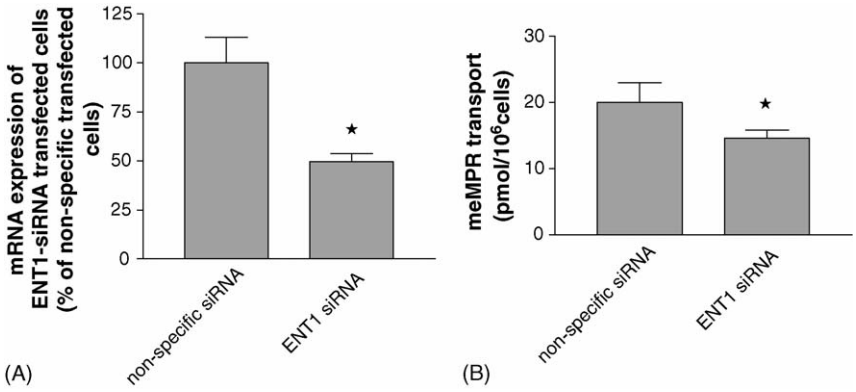

Fig. 5 – Reduction of the level of expression of ENT1 mRNA following transfection with siRNAs designed to target distinct sites in the ENT1 gene (A) is accompanied by a reduction in meMPR uptake (B) by wild-type MOLT4 cells. Cells transfected with non-specific siRNA were used as the control. mRNA and transport of meMPR were assayed 24 h and three days, respectively, after transfection. Intracellular levels of meMPR and meTIMP were determined by HPLC and are expressed as pmol/million cells. The values presented are the means ± S.D. of three independent experiments, $^{*}p < 0.05$.

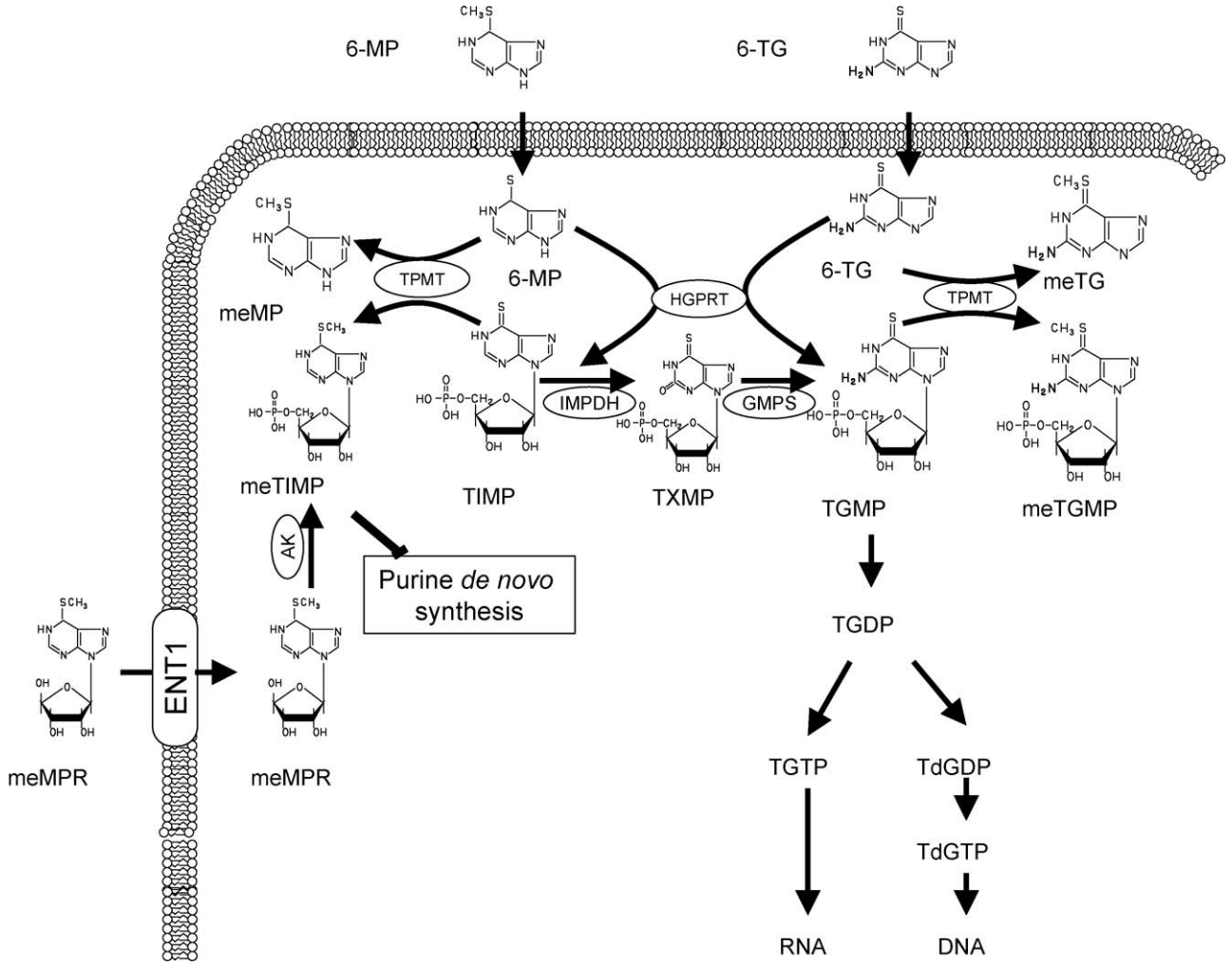

Fig. 6 – Intracellular metabolism of thiopurines. After entry into the cell, meMPR undergoes phosphorylation to meTIMP, which inhibits DNPS. 6-MP and 6-TG are converted by HGPRT and subsequently incorporated into DNA or RNA as TGN, or methylated by TPMT to inactive forms (meMP and meTG) or, in the case of 6-MP, to active meTIMP. HGPRT, hypoxanthine guanine phosphoribosyl-transferase; TPMT, thiopurine methyltransferase; IMPDH, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase; meMP, methyl mercaptopurine; meTG, methyl thioguanine; TIMP, 6-thioinosine 5′-monophosphate; TGMP, 6-thioguanosine 5′-monophosphate; TGN, thioguanine nucleotide.

enzyme were killed selectively by meMPR. Thus, the significant reduction in the rate DNPS observed in our resistant cells may make them hypersensitive to the potent inhibition of purine biosynthesis caused by meMPR. The reduction of intracellular nucleotide pools in these resistant cell lines to less than 50% of the normal levels may reflect less pronounced salvage of nucleosides. Accordingly, the enhanced sensitivity of our two resistant cell lines to meMPR can be explained, at least in part, by the fact that the uptake of this compound is normal, at the same time as the reduced intracellular nucleotide pools diminish the tolerance of these sublines to inhibition of *de novo* purine biosynthesis.

To further emphasize this situation, we calculated the ratio of meMPR uptake to intracellular levels of ATP and GTP versus the IC_{50} values for meMPR for our cell lines (data not shown). The higher level of meTIMP in relationship to intracellular levels of ATP and GTP in 6-MP-resistant cells again demonstrates that the cytotoxicity of meTIMP is related to both the level of meMPR (which is correlated to the extent of DNPS inhibition) and to depletion of purine nucleotides (which restricts the ability of the cells to cope with such inhibition). meTIMP, the phosphorylated metabolite of meMPR, is responsible for the toxicity observed with meMPR and this conversion is entirely dependent on the activity of adenosine kinase. Consequently, reduction in adenosine kinase activity is a well-known mechanism of resistance to meMPR [4]. However, our resistant cell lines exhibit normal levels of this activity.

In light of the fact that meMPR is a structural analogue of adenosine, nucleoside transporters would be expected to be involved in cellular uptake of this metabolite of 6-MP. In connection with our efforts to identify the route of meMPR transport into wild-type MOLT4 cells, Na^+ ions were found to have no influence on this transport, thereby ruling out the involvement of Na^+-ion dependent CNTs. In contrast, 10 nM NBTI or dipyridamole did inhibit the uptake of meMPR (also at a final concentration of 10 μM) by >30% (Fig. 4), providing evidence for the involvement of the first member of the ENT family, which is highly sensitive to these inhibitors. Since ENT1 transports nucleosides, but not nucleobases [13], the lack of effect of nucleobases on meMPR into our wild-type MOLT4 cells supports this conclusion. Furthermore, 1 mM adenosine inhibited the initial uptake of meMPR strongly (>85%, $p < 0.05$), as would be expected if they share the same transporter. In addition, silencing of the ENT1 gene by specific siRNA species resulted in a significant decrease in the uptake of meMPR.

The adaptive mechanisms that allow resistant cells to tolerate the toxic effects of a particular agent may actually enhance the effects of other drugs with different mechanisms of action. Accordingly, an improved understanding of mechanisms of resistance may help clinicians to design more individualized treatments. The fact that 6-MP and meMPR are transported into cells via different routes, together with the enhanced sensitivity of 6-MP- and 6-TG-resistant cells to meMPR (Fig. 6) may indicate that administration of meMPR or its analogues to patients experiencing relapse or resistance following thiopurine therapy could be useful. Clearly, however, this hypothesis requires further investigation.

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REFERENCES

[1] Elgemeie GH. Thioguanine, mercaptopurine: their analogs and nucleosides as antimetabolites. Curr Pharm Des 2003;9:2627–42.
[2] Fotoohi AK, Lindqvist M, Peterson C, Albertioni F. Involvement of the concentrative nucleoside transporter 3 and equilibrative nucleoside transporter 2 in the resistance of T-lymphoblastic cell lines to thiopurines. Biochem Biophys Res Commun 2006;343:208–15.
[3] Vogt MH, Stet EH, De Abreu RA, Bokkerink JP, Lambooy LH, Trijbels FJ. The importance of methylthio-IMP for methylmercaptopurine ribonucleoside (Me-MPR) cytotoxicity in Molt F4 human malignant T-lymphoblasts. Biochim Biophys Acta 1993;1181:189–94.
[4] Yamanaka H, Kamatani N, Nishida Y, Nishioka K, Mikanagi K. Relationship between phosphorylation and cytotoxicity of 2-chloroadenosine and 6-methylmercaptopurine riboside in human cells. Biochim Biophys Acta 1984;798:291–4.
[5] Wiemann MC, Crabtree GW, Weitberg AB, Spremulli EN, Cummings FJ, Murray C, et al. Phase I trial of the combination of 6-methylmercaptopurine riboside and 5-fluorouracil. Med Oncol Tumor Pharmacother 1988;5:113–6.
[6] Hageboutros A, Hudes GR, Greene F, LaCreta FP, Brennan J, O'Dwyer PJ. Phase I trial of fluorouracil modulation by N-phosphonacetyl-L-aspartate and 6-methylmercaptopurine ribonucleoside (MMPR), and leucovorin in patients with advanced cancer. Invest New Drugs 1997;15:139–45.
[7] Redei I, Green F, Hoffman JP, Weiner LM, Scher R, O'Dwyer PJ. Phase II trial of PALA and 6-methylmercaptopurine riboside (MMPR) in combination with 5-fluorouracil in advanced pancreatic cancer. Invest New Drugs 1994;12:319–21.
[8] Masson E, Synold TW, Relling MV, Schuetz JD, Sandlund JT, Pui CH, et al. Allopurinol inhibits de novo purine synthesis in lymphoblasts of children with acute lymphoblastic leukemia. Leukemia 1996;10:56–60.
[9] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[10] Plunkett W, Hug V, Keating MJ, Chubb S. Quantitation of 1-beta-D-arabinofuranosylcytosine 5′-triphosphate in the leukemic cells from bone marrow and peripheral blood of patients receiving 1-beta-D-arabinofuranosylcytosine therapy. Cancer Res 1980;40:588–91.
[11] Inaba M, Fukui M, Yoshida N, Tsukagoshi S, Sakurai Y. Collateral sensitivity of 6-mercaptopurine-resistant sublines of P388 and L1210 leukemia to the new purine antagonists, 5-carbamoyl-1H-imidazol-4-yl piperonylate and 4-carbamoylimidazolium 5-olate. Cancer Res 1982;42:1103–6.
[12] Li W, Su D, Mizobuchi H, Martin DS, Gu B, Gorlick R, Cole P, Bertino JR. Status of methylthioadenosine phosphorylase and its impact on cellular response to L-alanosine and methylmercaptopurine riboside in human soft tissue sarcoma cells. Oncol Res 2004;14:373–9.
[13] Baldwin SA, Beal PR, Yao SY, King AE, Cass CE, Young JD. The equilibrative nucleoside transporter family, SLC29. Oflugers Arch 2004;447:735–43.