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Thiopurine Metabolism and Identification of the Thiopurine Metabolites Transported by MRP4 and MRP5 Overexpressed in Human Embryonic Kidney Cells

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ABSTRACT

Mercaptopurines have been used as anticancer agents for more than 40 years, and most acute lymphoblastic leukemias are treated with 6-mercaptopurine (6MP) or 6-thioguanine (TG). Overexpression of the two related multidrug resistance proteins MRP4 and MRP5 has been shown to confer some resistance against mercaptopurines, which has been attributed to extrusion of mercaptopurine metabolites by these transporters. We have analyzed the mercaptopurine metabolites formed in human embryonic kidney cells and determined which metabolites are extruded by MRP4 and MRP5. Incubation with 6MP led to the formation of thioinosine and thioxanthosine metabolites and we found that thio-IMP was transported by both MRP4 and MRP5; MRP5 showed the highest transport rate. In contrast, only MRP5 transported thioxanthosine monophosphate (tXMP). During incubation with TG, the monophosphorylated form of thioguanosine was transported by both MRP4 and MRP5; the highest transport rate was for MRP4. Similarly, only 6-methyl-thio-IMP was formed during incubation with 6-methyl mercaptopurine riboside. This compound was a substrate for both MRP4 and MRP5; MRP4 showed the highest transport rate. Our results show that all major thiopurine monophosphates important in the efficacy of mercaptopurine treatment are transported by MRP4 and MRP5, although the substrate specificity of the two transporters differs in detail.

Multidrug resistance protein 4 (MRP4/ABCC4) and multidrug resistance protein 5 (MRP5/ABCC5) belong to the MRP family of multispecific drug transporters (reviewed in Hipfner et al., 1999; Borst et al., 2000). This family now consists of nine members,[1] MRP1–9, which are also called ABCC1–6 and 10–12, respectively. Amino acid sequence comparisons show homology between the MRPs and the multidrug transporter P-glycoprotein. According to secondary structure predictions, MRP1–8 contain two nucleotide binding domains and at least 12 transmembrane segments, the so-called P-glycoprotein–like core, that form the essential transporter. They are mainly localized in the plasma membrane and show ATP-dependent transport of a broad range of compounds. Most MRP substrates are organic anions including many drugs conjugated to glutathione, glucuronide, or sulfate. Transport of nonanionic compounds such as vincristine and doxorubicin is also found in some cases, but this requires (cotransport with) glutathione (Zaman et al., 1995; Versantvoort et al., 1995). Because many MRP substrates are drugs used in cancer therapy, the overexpression of MRPs in cancer cells may contribute to resistance of such cells.

Like the other MRPs, MRP4 and MRP5 are organic anion transporters (Schuetz et al., 1999; McAleer et al., 1999; Wijnholds et al., 2000; Chen et al., 2001), but they have the unique ability to transport nucleoside monophosphate analogs (Schuetz et al., 1999; Jedlitschky et al., 2000; Lee et al.,

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P.R.W. and G.R. contributed equally to this study.

[1] For an overview of the ABC-family members and nomenclature, see http://nutrigene.4t.com/humanabc.htm.

ABBREVIATIONS: MRP, multidrug resistance protein; 6MP, 6-mercaptopurine; TG, 6-thioguanine; tNMP, thionucleoside monophosphate; tIMP, thio-IMP; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; PRPP, 5-phosphoribosyl-1-pyrophosphate; tGMP, thio-GMP; NMP, nucleoside monophosphate; TPMT, thiopurine methyltransferase; MeMP, 6-methyl-mercaptopurine; MetIMP, 6-methyl-thio-IMP; HEK, human embryonic kidney; tXMP, thioxanthosine monophosphate; TGrib, thioguanine riboside; MPrib, 6-mercaptopurine riboside; TX, thioxanthine; TXrib, thioxanthine riboside; tGTP, thio-GTP; mAb, monoclonal antibody; PBS, phosphate-buffered saline; UV_{max}, ultraviolet absorbance maximum; HPLC, high-performance liquid chromatography.

2000; Wijnholds et al., 2000; Chen et al., 2001) such as the antiviral compound 9-(2-phosphonylmethoxyethyl)adenine. The physiological substrates of these transporters may be cAMP and cGMP (Jedlitschky et al., 2000; Chen et al., 2001). Overexpression of MRP5 (Wijnholds et al., 2000) and MRP4 (Chen et al., 2001) in cells results in resistance to 6-mercaptopurine (6MP) and 6-thioguanine (TG). Both 6MP and TG are purine nucleobase analogs with a sulfur at the C-6 position. 6MP was first synthesized in 1952 and is an important drug in the treatment of acute lymphoblastic leukemia, especially in children (Elion et al., 1952; Burchenal et al., 1953). In addition, 6MP is used as an immunosuppressant [for example, in the treatment of Crohn's disease (Elion, 1989; Markowitz et al., 2000)]. The guanine analog TG, synthesized after 6MP, has a type of action similar to that of 6MP (Elion, 1989; LePage, 1963; Lowe et al., 2001). 6MP and TG are prodrugs and their toxicity depends on their intracellular conversion to thionucleoside monophosphates (tNMPs). Thiopurine metabolism is as extensive as the metabolism of normal purines (Zimm et al., 1985; Martin, 1987; Elion, 1989; Keuzenkamp-Jansen et al., 1995; Aubrecht et al., 1997). After uptake, 6MP is converted into thio-IMP (tIMP) by hypoxanthine-guanine phosphoribosyl transferase (HGPRT), with phosphoribosyl pyrophosphate (PRPP) as the phosphoribosyl donor. In a similar way, TG can be converted into thio-GMP (tGMP). tIMP can be converted into tGMP in two steps: first, thioxanthosine monophosphate (tXMP) is formed by IMP dehydrogenase; second, tGMP is formed by GMP synthetase. tGMP can, in turn, be further phosphorylated to tGTP, which can be incorporated into RNA or, after reduction of the ribose moiety, into DNA. Alternatively, thiopurine methyltransferase (TPMT) can methylate 6MP to form the metabolic end product 6-methyl-MP (MeMP), thereby reducing the toxicity of 6MP (Lennard et al., 1987). Conversely, methylation of tIMP yields 6-methyl-tIMP (MetIMP), an inhibitor of de novo purine synthesis (Vogt et al., 1993). Several steps in purine metabolism have been reported to be involved in resistance of acute lymphoblastic leukemia cells to 6MP: a reduced nucleotide formation caused by low HGPRT activity or low PRPP levels (Rosman et al., 1974; Zimm et al., 1986; Vogt et al., 1993); increased dephosphorylation of the tNMPs by alkaline phosphatase or 5′-nucleotidase (Rosman et al., 1974; Zimm et al., 1986; Pieters et al., 1992; Vogt et al., 1993); and increased methylation of 6MP (Relling et al., 1999).

Because both MRP4 and MRP5 are ATP-dependent efflux pumps, a possible explanation for the resistance observed in cells overexpressing these proteins is the increased removal of an essential thiopurine metabolite from the cells. After preloading cells with radiolabeled 6MP under ATP-depleting conditions, Wijnholds et al. (2000) found that cells overexpressing MRP5 showed an increased efflux of the radiolabel. In similar experiments Chen et al. (2001) found a similar increased efflux for cells overexpressing MRP4. Analysis of the efflux medium showed that several compounds were preferentially effluxed by the MRP5-overexpressing cells, one of which coeluted with tIMP. For MRP4, no attempt was made to identify the effluxed metabolites.

The aim of the current study was to generate a comprehensive picture of the intracellular thiopurine metabolism in HEK293 cells under continuous exposure to thiopurines. We analyzed the metabolites excreted from parental HEK293 as well as from cells overexpressing either MRP4 or MRP5 to compare the substrate spectra of MRP4 and MRP5 and exclude the possibility that modified thiopurine metabolism was involved in thiopurine resistance. To this end, we set up a detection system to investigate the metabolism of thiopurines in HEK293 cells and to identify the metabolites excreted. Upon exposure to the thiopurine nucleobases 6MP and TG, or to 6-methyl-mercaptopurine riboside (MeMPrib), we found that tNMPs are exported by both MRP4 and MRP5 with overlapping substrate specificity. However, only MRP5-overexpressing cells transported tXMP, whereas MRP4-overexpressing cells showed the higher rates of transport of tGMP and MetIMP.

Experimental Procedures

Chemicals. Poly-D-lysine, 6MP, TG, thioxanthine (TX), 6-thioguanosine (TGrib), 6-mercaptopurine riboside (MPrib), MeMP, 6-methyl thioguanine, MeMPrib, and PRPP were obtained from Sigma (Zwijndrecht, The Netherlands). tIMP, tGMP, and tXMP were synthesized from 6MP, TG, TX, and PRPP using HGPRT from *Saccharomyces cerevisiae* (Sigma), as described previously (Wijnholds et al., 2000). MetIMP and 6-methylated tGMP were made from tGMP or tIMP (1 mM) by overnight incubation at room temperature in an aqueous solution containing 70 mM methyl bromide (Sigma) and 3.75% ammonia; thereafter, excess ammonia and methyl bromide were evaporated under vacuum, essentially as described previously (Keuzenkamp-Jansen et al., 1995). TXrib was generated from tXMP by 5′-nucleotidase, and thio-GDP, tGTP, thio-IDP, and thio-ITP were synthesized as described previously (Breter and Mertes, 1990).

Cell Lines. HEK293 parental cells and HEK293/5I and HEK293/5E cells transduced with MRP5 cDNA have been described previously (Wijnholds et al., 2000). The subclone HEK293/5GE was isolated at the same time as HEK293/5I and HEK293/5E but has not been described before. HEK293/5I and HEK293/5E have high MRP5 overexpression, whereas HEK293/5GE has a moderate increase in MRP5 relative to the parental cells (see *Results*). MRP4-overexpressing cells HEK293/4.3 and HEK293/4.63 were made by transducing HEK293 cells with pMSCV-IRES-EGFP virus containing full-length MRP4 cDNA, the cloning of which has been described elsewhere (Adachi et al., 2002). All cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37°C under 5% CO_2/humidified air. The cells were routinely checked for mycoplasma and MRP4 and MRP5 expression levels.

Monoclonal Antibodies. Anti-MRP5 monoclonal antibody (mAb) NKI-12C5 was generated by cloning a fragment of the mouse Mrp5 cDNA, encoding amino acids 1 to 38, into the pMAL-c vector (New England Biolabs, Beverly, MA) to produce a fusion protein consisting of *Escherichia coli* maltose-binding protein fused to the N terminus of mouse Mrp5. The affinity-purified protein was injected into rats, and hybridoma cells producing MRP5-specific mAbs were generated, essentially as described previously (Harlow and Lane, 1988). NKI-12C5 specifically recognizes the full-length human MRP5 and mouse Mrp5 protein. The MRP4-specific antibody NKI-12C4 was generated in a similar way using a maltose-binding protein fusion protein containing an internal epitope (amino acids 372–432) from human MRP4. A more complete characterization of these mAbs will be presented elsewhere.

Western Blot Analysis. Protein from a total cell lysate was quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA), fractionated on a 8% polyacrylamide slab gel and transferred to a nitrocellulose membrane by electroblotting, essentially as described previously (Kool et al., 1997). After blocking for 1 h in phosphate-buffered saline (PBS) containing 1% nonfat dry milk, 1% bovine serum albumin, and 0.05% Tween 20, the membrane was incubated for 1 h at room temperature with the first antibody. As secondary

antibody, horseradish peroxidase-labeled goat anti-rat antibody, preabsorbed for both mouse and human IgGs, was used at a dilution of 1:2000 (Santa Cruz Technology, Santa Cruz, CA). Enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was used for detection.

Immunohistochemistry. To detect MRP4 and MRP5 by immunofluorescence, cells were grown overnight on poly(D-lysine)-coated microscope slides. The next day, the cells were fixed with methanol (30 s, −20°C) and immunostained with NKI-12C4 and NKI-12C5 (undiluted hybridoma culture medium). The first antibody was visualized using a goat anti-rat-Alexa488-conjugated fluorescent antibody (1:500; Molecular Probes, Leiden, the Netherlands). Cells were mounted using Vectashield (Vector Laboratories, Burlingame, CA) containing 40 μg/ml ethidium bromide to visualize nuclear DNA.

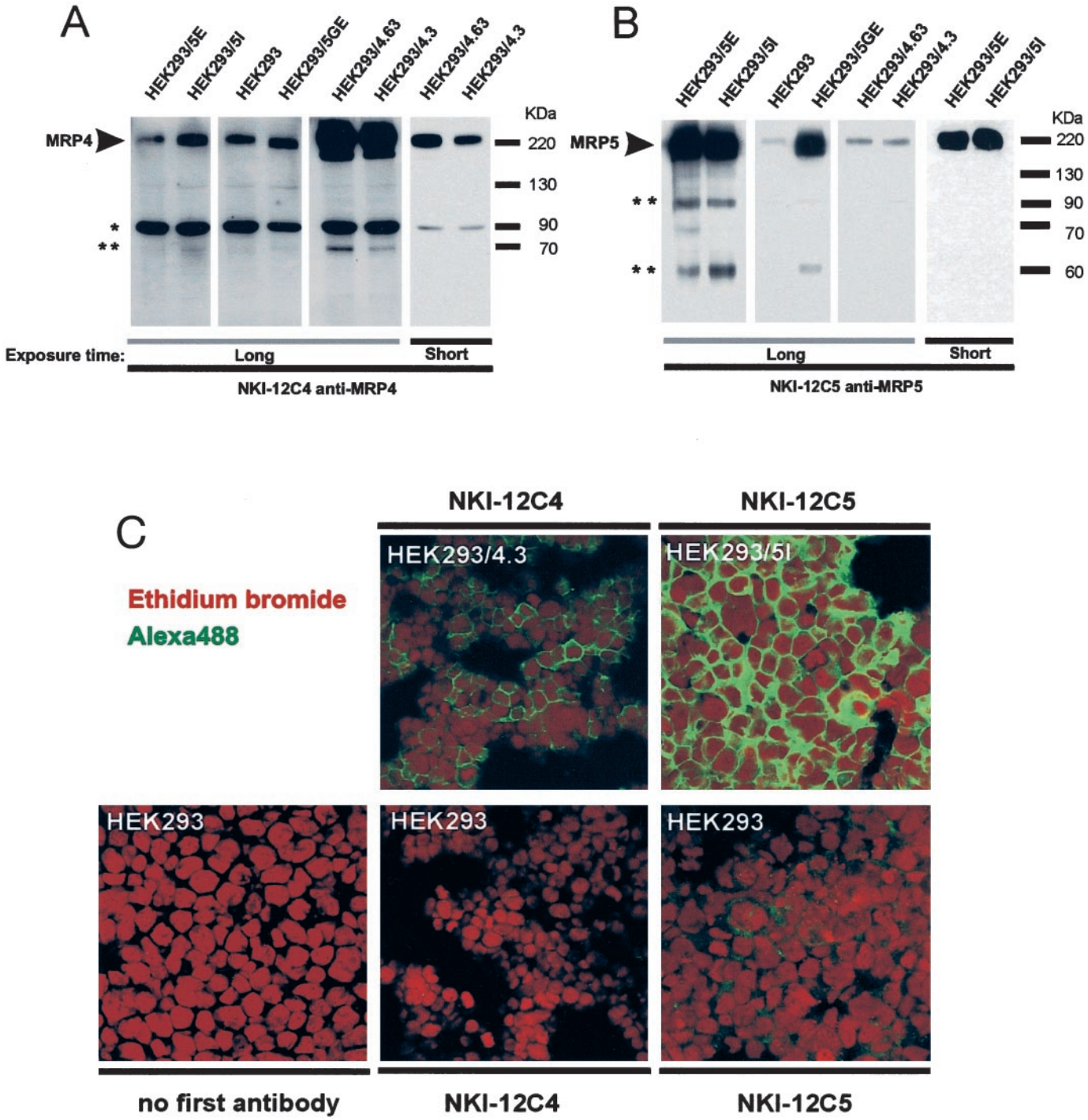

Fig. 1. Western blot analysis and immunofluorescent detection of MRP4 and MRP5. MRP4 and MRP5 expression in the three cell lines used in this study is shown. For the Western blot, 15 μg of total protein was loaded per lane. MRP4 was detected by rat anti-human MRP4 monoclonal antibody NKI-12C4 (A) and MRP5 by rat anti-mouse MRP5 monoclonal antibody NKI-12C5 (B). Detection of MRP4 and MRP5 by immunofluorescence (C) was done on methanol-fixed cells using the indicated antibodies as outlined under *Materials and Methods*. MRP4- and MRP5-specific staining is shown in green, and the nuclear DNA stained by ethidium bromide is shown in red. *, ~90 kDa band recognized by NKI-12C4 found in all cell lines; thus, it is most probably an endogenous protein. **, ~75 kDa band in A, two bands of ~95 and ~60 kDa in B, of which the intensity increases with increasing MRP4 or MRP5 expression. The shorter film exposures demonstrate the relative levels of the introduced protein in the MRP4- and MRP5-overexpressing cells.

Slides were analyzed by confocal laser-scanning microscopy (Leica, Heidelberg, Germany), using a 488-nm laser for excitation and a 530-nm band-pass filter for detection of Alexa488 and a 560-nm high-pass filter for detection of ethidium bromide-stained nuclear DNA.

Cytotoxicity. Relative growth of cells in the presence of thiopurines was tested essentially as described before (Wijnholds et al., 2000). In short, cells were plated in triplicate in 96-well plates (1500 cells/well, 100 μl/well) in conditioned medium. The next day, drug was added at the appropriate dilutions in a volume of 25 μl. After an additional 4 days, cell growth was determined using the CyQuant cell proliferation assay kit (Molecular Probes). The fluorescence in each well was determined using a CytoFluor 4000 plate reader (Applied Biosystems, Foster City, CA) and the IC_{50} value was defined as the concentration of drug that inhibited growth of the cells by 50%. The relative resistance was then determined as the ratio of the IC_{50} values of the transduced and parental cells.

Transport Experiments. Cells were seeded at a density of 2×10^6 per well in poly(D-lysine)-coated 12-well plates and were grown overnight. The next day, the cells were washed with PBS and then incubated at 37°C with various thiopurines in Hanks' buffered salt solution (Invitrogen). At the time points indicated, both incubation medium and the cells were collected. The incubation medium was centrifuged and the supernatant was used for analysis. For the analysis of the intracellular metabolites, the cells were washed with ice-cold PBS and extracted using 70% methanol/water, and insoluble material was removed by centrifugation. After complete evaporation of the methanol/water phase at 40°C, the residue was solubilized in 200 μl of HPLC elution buffer A (see below) and analyzed by HPLC.

HPLC Analysis. Cell extracts and incubation media were analyzed by reversed-phase ion-pairing HPLC analysis using a Luna-C18 column (284 Luna, 5 μm, 250 × 4.6 mm; Phenomenex, Cheshire, UK) and a two-buffer gradient system to separate the different nucleotides. The following pump conditions were used: 0–5 min, 100% buffer A; 5–25 min, linear to 70% buffer B; 25–28 min, 70% buffer B; 28–30 min, linear to 100% buffer A; 30–45 min, 100% buffer A. The composition of buffer A was 100 mM NaH_2PO_4, 5.9 mM tetrabutylammonium hydrogen sulfate, 0.34 mM EDTA, and 1% (v/v) acetonitrile. Buffer B was the same as buffer A but contained 25% (v/v) acetonitrile. The flow was kept at 1 ml/min. The complete UV-visible absorbance spectrum (200–800 nm) of the effluent was monitored using a Waters 966 photodiode array detector (Waters Chromatograpy B.V., Etten-Leur, the Netherlands). The metabolites were identified by their specific retention times and UV absorbance maxima (UV_{max}; see Table 2). Thiopurine metabolites were quantified at their UV_{max} by relating the peak area of the metabolites detected with the peak area measured for a standard amount of either 6MP, TG, TX, or MeMPrib. Intracellular ATP was monitored to ensure that the cells were metabolically active, and the absence of extracellular ATP confirmed that cells had remained intact during the experiment.

Results

MRP4 and MRP5 Levels in Transfected HEK293 Cells. The levels of MRP4 and MRP5 in the HEK293 cell lines transduced with MRP4 or MRP5 cDNA, respectively, were quantified using two new mAbs, NKI-12C4 (MRP4) and NKI-12C5 (MRP5), as shown in Fig. 1. Recombinant MRP4 protein migrates as a broad band of approximately 220 to 230 kDa; MRP4-transduced HEK293/4.3 and HEK293/4.63 cells contain similar levels of MRP4 (Fig. 1A). NKI-12C4 recognizes an endogenous cross-reacting protein of ~90 kDa in all HEK293 variants, in addition to a band of ~70 kDa, which increases in intensity with increasing MRP4 expression. The latter may represent an MRP4 breakdown product. MRP5 migrates as a single band of 220 to 230 kDa (Fig. 1B). Overexpression of MRP5 is high in HEK293/5I and HEK293/5E and intermediate in HEK293/5GE cells. Two smaller bands of ~95 and ~60 kDa may represent breakdown products. In contrast to our previous anti-MRP5 antibody (Wijnholds et al., 2000), the new antibody NKI-12C5 clearly detected endogenous expression of MRP5 in the parental HEK293 cells, whereas the NKI-12C4 mAb detects endogenous MRP4 (Fig. 1, A and B). We see no effect on endogenous levels after introduction of exogenous MRP4 or MRP5 (Fig. 1, A and B). Figure 1C shows the location of MRP4 and MRP5 in HEK293 cells with immunofluorescence. Although we detect weak signals for endogenous MRP4 and MRP5 in the parental line, the intensity was too low to determine the subcellular localization (Fig. 1C).

Overexpression of MRP5 in HEK293 cells, as in HEK293/5I cells, results in a 2- to 3-fold relative resistance against 6MP and TG (Table 1). Analogous results were obtained for HEK293 cells overexpressing MRP4, as shown in Table 1.

HPLC Analysis. To analyze the thiopurine metabolites formed by the HEK293 cells, we set up a reversed-phase ion-pairing HPLC system in combination with spectral UV-visible detection. This system is stable and allows the separation of the metabolites of both physiological and thiopurine nucleosides within 30 min (Fig. 2). The combination of elution time and UV-visible absorbance spectra (Fig. 2, insets) makes it possible to discriminate between metabolites. Table 2 provides an overview of UV_{max} and retention times for the metabolites analyzed in this study.

6-Mercaptopurine Uptake by HEK293 Cells. In previous experiments with HEK293/5I cells (Wijnholds et al., 2000), cells were loaded with radiolabeled 6MP under ATP-depletion conditions and efflux was followed after restoration of cellular ATP. This discontinuous incubation is somewhat artificial and results in 10-fold lower 6MP uptake than in the presence of normal cellular ATP levels. We therefore switched to a protocol in which the cells are continuously exposed to 6MP under normal conditions. 6MP is lipophilic and is assumed to rapidly equilibrate across the plasma membrane. Accumulation of 6MP is therefore mainly determined by its intracellular ribophosphorylation, a process that is dependent on HGPRT activity and the intracellular PRPP concentration. Uptake of 6MP by HEK293 cells over 4 h increased with increasing 6MP (Fig. 3A). However, increasing the 6MP concentration 5-fold from 2.5 to 12.5 μM yielded only a 2-fold increase in uptake. This suggests that the uptake of 6MP was no longer linear (Fig. 3B), presumably because the cells were unable to maintain the initial rate of conversion of 6MP into tIMP. HEK293, HEK293/5I, and HEK293/4.3 cells took up similar levels of 6MP from the medium (Fig. 3).

TABLE 1

Relative resistance of HEK293 cells overproducing MRP4

Values are the concentration at which the growth inhibition was 50% of the maximum growth (IC_{50}). The relative resistance is calculated as the IC_{50} ratio of the transduced cells and the parental cells. Data are the mean (± S.E.M.) of at least three experiments.

	IC_{50}	Relative Resistance			
	HEK293	HEK293/5I	HEK293/5E	HEK293/4.3	HEK293/4.63
	μM				
6MP	5.2 ± 0.9	3.1[a]	2.5[a]	2.7 ± 0.4	5.6 ± 2.4
TG	1 ± 0.2	2.1[a]	2.3[a]	2.6 ± 0.6	4.1 ± 0.1

[a] Wijnholds et al. (2000).

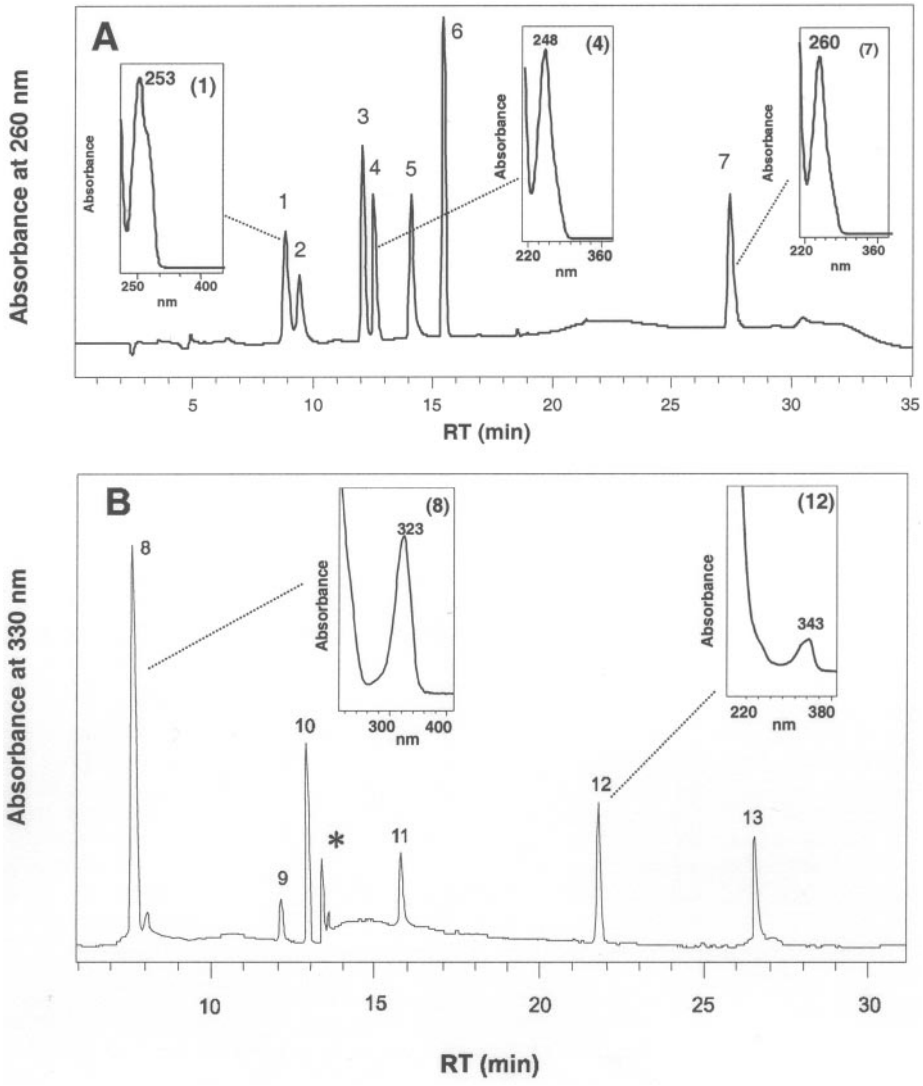

Fig. 2. HPLC analysis of reference compounds. Chromatograms of mixtures of nucleobases, nucleosides and nucleotides separated by reversed-phase ion-pairing HPLC. A, chromatogram showing the separation of guanosine (1), inosine (2), IMP (3), GMP (4), adenosine (5), AMP (6), and ATP (7), detected at 260 nm. B, chromatogram showing the separation of 6MP (8), TX (9), MPrib (10), tIMP (11), TXrib (12), and tXMP (13), detected at 330 nm. *, peaks that were derived from the Hanks' balanced salt solution in which thiopurines were dissolved. UV absorbance is given in arbitrary units. The insets show examples of absorbance spectra of indicated peaks, which were used to identify different metabolites.

TABLE 2
Ultraviolet absorbance maximum (UV_{max}) and the retention times in reversed HPLC analysis for several nucleobases, nucleosides, and nucleotides used in this study
Note that the UV_{max} values of the compounds indicated per row are the same.

	UV_{max}	Retention Times				
		Nucleobase	Nucleoside	NMP	NDP	NTP
	nm	*min*				
Thioguanines	342	8.2	13.2	16.1	22.3	29.2
Thioinosines	323	7.6	12.9	15.6	22.8	29.2
Thioxanthines	343	12.6	21.5	26.2	N.D.	N.D.
6-Methylated thioinosines	286	23.2	25.7	28.0	N.D.	32.6
Adenosines	260	N.D.	14.1	15.1	21.5	28.1

NMP, nucleoside monophosphate; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate; N.D., not determined.

Thiopurine Efflux Kinetics for HEK293 Cells in the Continuous Presence of 6-Mercaptopurine. Five thiopurine metabolites were present in the chromatograms from HPLC analysis of the medium from HEK293, HEK293/4.3, and HEK293/5I cells after incubation with 2.5 μM 6MP. These were identified as MPrib, tIMP, TX, TXrib, and tXMP. Figure 4 shows the excretion of the four major metabolites, MPrib, tIMP, TXrib and tXMP over time. Table 3 gives the excretion rates for the different thiopurines and shows that the excretion of tIMP was greatly increased in cells overexpressing MRP4 or MRP5. The excretion of tXMP was detected only with HEK293/5I cells. Using the other MRP4 and MRP5 overexpressing HEK293 clones (Fig. 1), we obtained similar results (data not shown).

To exclude the possibility that an increase in intracellular formation of tIMP and tXMP was responsible for the release of these compounds from HEK293/4.3 and HEK293/5I cells, the intracellular thiopurine metabolites were determined. The time course of metabolite formation in HEK293 cells is shown in Fig. 5. After incubation with 2.5 μM 6MP, intracel-

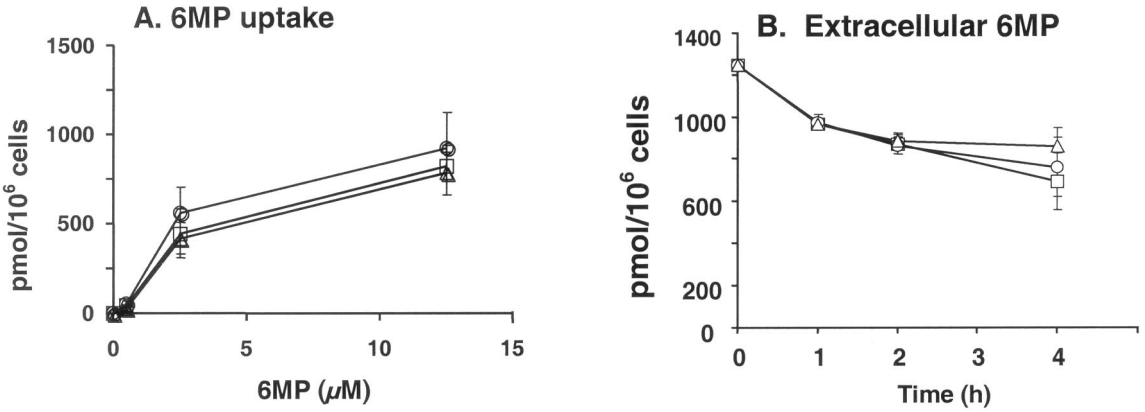

Fig. 3. Uptake of 6MP. A, concentration-dependent uptake of 6MP was determined after 4 h for the HEK293 (□), HEK293/5I (○), and HEK293/MRP4.3 (△) cells. Initial 6MP concentrations in the incubation medium were 0.5, 2.5, and 12.5 μM. After 4 h of incubation at 37°C, the 6MP concentration in the medium was determined. Uptake was determined by the difference in 6MP concentration in the medium at $t = 0$ and $t = 4$ h. B, time-dependent uptake of 2.5 μM 6MP, with symbols as in A. The extracellular 6MP concentration is plotted as picomoles per 10^6 cells calculated using the volume of incubation medium (1 ml) and number of cells (2×10^6). Data are the mean ± S.E.M. of three independent determinations.

lular tIMP levels rose rapidly (Fig. 5A), followed by a rise in MPrib. This suggests that the MPrib is made by dephosphorylation of tIMP by a phosphatase or nucleotidase (Skladanowski et al., 1996; Hunsucker et al., 2001), followed by rapid excretion of the nucleoside via a nucleoside transporter (Baldwin et al., 1999). As expected, intracellular tXMP and TXrib concentrations follow the rise in tIMP in the cells with some delay (Fig. 5), and these compounds also appear later in the medium (Fig. 4). The decrease in 6MP uptake after 1 h (Fig. 3B) is accompanied by a strong decrease in intracellular tIMP and MPrib (Fig. 5). This suggests that conversion of 6MP decreases sharply after 1 h of incubation. Eventually, excretion of tIMP also slows down after 2 h (Fig. 4A). Extracellular MPrib even tends to decrease after 2 h (Fig. 4C), which may be caused by re-entry of MPrib into the cells and conversion into 6MP. Indeed, when HEK293 cells were incubated with MPrib, substantial amounts of 6MP were formed (data not shown).

The bulk of the 6MP taken up by the cells was found back in the medium as nucleoside in the form of MPrib and to a lesser extent TXrib (Fig. 4). To exclude the possibility that these nucleosides were produced by extracellular dephosphorylation of exported nucleotides, we incubated cells in medium with tIMP (2.5 μM): only 1 to 1.5% tIMP/10^6 cells/h was dephosphorylated (results not shown), suggesting that the vast majority of MPrib observed was formed intracellularly.

The total tIMP accumulated after a 1-h incubation was 60, 50, and 60 pmol/10^6 cells for HEK293, HEK293/4.3, and HEK293/5I (Fig. 5). Although comparable intracellular tIMP levels were achieved, the rate of tIMP efflux (calculated by simple linear regression) was markedly different: 0.09, 0.56 and 0.64 pmol/10^6 cells/min for HEK293, HEK293/4.3, and HEK293/5I cells, respectively (Table 3). Only the HEK293/5I seemed to efflux tXMP (Fig. 4B and Table 3) at a rate comparable with the rate of tIMP excretion (Table 3). The levels of tIMP and tXMP accumulated after 2 h were decreased in the resistant cells (Fig. 5), showing that as tIMP production slows, the pumps are able to decrease tNMP levels in the cells. With HEK293/4.63 cells, we obtained results similar to those of the HEK293/4.3 cells, and the HEK293/5I results were essentially duplicated with HEK293/5E and HEK293/5GE cells (data not shown). The tNMP transport activity of the HEK293/5GE cells was lower, however, than that of the HEK293/5I cells, in line with their lower expression (Fig. 1). Both HEK293/4.3 (Fig. 4D) and HEK293/4.63 cells (not shown) excreted more TXrib than the HEK293 parental and MRP5-overexpressing cells. This was not caused by an increased excretion of thionucleosides in general, because the MPrib excretion was lower in these cells (Fig. 4C).

In our 6MP incubation experiments, we detected neither thioguanine metabolites nor di- or triphosphorylated thiopurine nucleosides. Methylated thioinosines (UV_{max} ~286 nm) or methylated thioguanosines (UV_{max} ~310 nm) were not detected in cells either. Absence of thioguanine nucleotides and methylated thiopurines might be attributable to the low rates of TPMT and IMP dehydrogenase under our conditions. Small amounts of di- and triphosphates are probably formed but remain undetectable because our cell extracts contain high background absorbance in the area where these compounds elute from the HPLC. It is unlikely, however, that we miss a substantial fraction of the metabolites formed, because we obtain nearly 100% recovery of the 6MP taken up in the form of 6MP metabolites in the cells plus extracellular medium. The calculated values for the 4-h time points were 94 ± 3, 96 ± 14, and 103 ± 25% (mean ± S.E.M.) for HEK293, HEK293/4.3, and HEK293/5I cells, respectively.

Thiopurine Metabolism and Transport by HEK293 Cells Incubated with 6-Thioguanine. Because we were unable to detect TG metabolites in cells incubated with 6MP, we tested TG itself, a compound to which the transfectants are resistant (Table 1). The HEK293 cells took up TG at about the same rate as 6MP (results not shown). The only metabolites found consistently were tGMP and TGrib. The excretion of tGMP was increased in both MRP4 and MRP5 transfectants; the increase was most pronounced in the MRP4 cells (Fig. 6A and Table 3). The efflux rates were 0.24, 0.77, and 1.22 pmol/10^6 cells/min for HEK293, HEK293/5I, and HEK293/4.3 cells, respectively. Excretion of TGrib was also substantial and did not differ for the three cell lines (Table 3 and Fig. 6B).

In contrast to the intracellular MPrib levels formed during exposure to 6MP, intracellular TGrib levels stayed relatively low and did not show a high peak after 1 h (Fig. 6D). In the

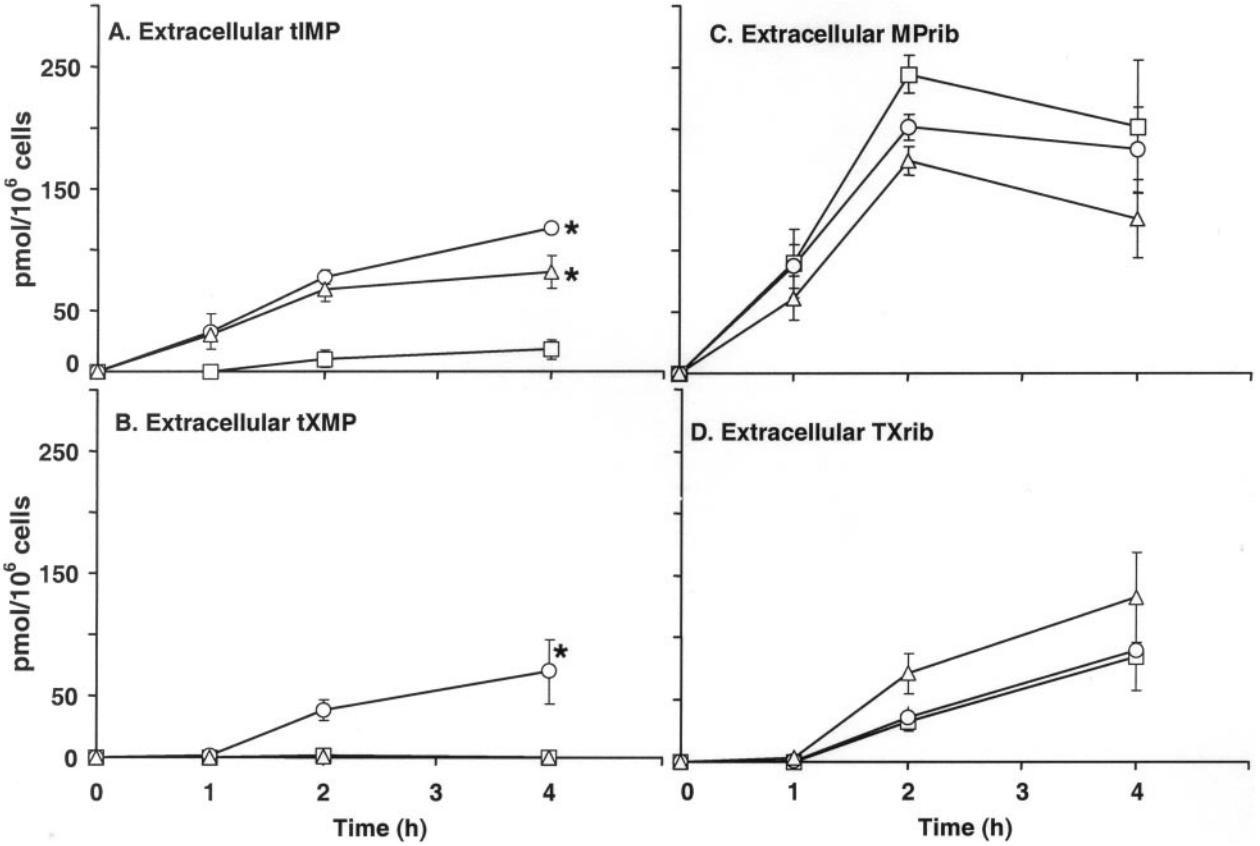

Fig. 4. 6MP metabolites excreted by HEK293 cells. Metabolites excreted from the HEK293 (□), HEK293/5I (○), and HEK293/4.3 (△) during continuous incubation with 6MP (initial concentration 2.5 μM). The levels of tIMP (A), tXMP (B), MPrib (C), and TXrib (D) were quantified at their UV_{max} wavelength, using chromatograms similar to those shown in Fig. 2. The amounts of the various thioinosine and thioxanthosine metabolites were calculated by relating the peak area of the detected metabolites with the peak area obtained with standard amounts of either 6MP or TX. The data are the mean ± S.E.M of three independent determinations. The indicated data points were significantly different (*, $p < 0.05$) from the parental cells in a Student's t test.

TABLE 3

Rate of thiopurine metabolite excretion for HEK293 cells

The excretion rates for the different metabolites formed in HEK293 cells after incubation with the thiopurine precursors 6MP, TG and MeMPrib (see text for details). Slopes were calculated by a simple linear regression using those time points for which the rate was relatively constant.

	6MP					TG		MeMPrib
	tIMP[a]	tXMP[b]	MPrib[a]	TXrib[b]	TX[b]	tGMP[c]	TGrib[c]	MetIMP[c]
	pmol / 10^6 cells / min							
HEK293	0.09	0.00	2.04	0.46	0.33	0.24	1.02	1.3
HEK293/4.3	0.56	0.00	1.45	0.69	0.13	1.22	0.78	3.2
HEK293/5I	0.64	0.36	1.68	0.49	0.11	0.77	1.00	1.9

[a] Rate was calculated for the 0- to 2-h time points.
[b] Rate was calculated for the 1- to 4-h time points.
[c] Rate was calculated for the 0- to 4-h time points.

analysis of the intracellular fractions, di- and triphosphorylated thioguanines were detected in only one of three experiments. In that experiment, we found a peak after 1 h with a retention time of 29 min and UV_{max} of ~342 nm, which is most probably tGTP. This was present at levels of 51, 33, and 39 pmol/10^6 cells for the HEK293, HEK293/4.3, and HEK293/5I cells, respectively, comparable with the tGMP levels of these cells. The overall recovery of TG taken up by the cells for the 4 h time point was 86 ± 19, 109 ± 11, and 106 ± 9% (mean ± S.E.M.). We found no thioxanthosine metabolites, showing that the HEK293 cells do not deaminate tGMP and TGrib at measurable rates under our conditions.

Thiopurine Transport from Cells Incubated with 6-Methyl-mercaptopurine Riboside. In patients treated with 6MP, 6-methylated thiopurines are formed by TPMT. 6MP can be methylated to MeMP, which is not a substrate of HGPRT. In this case, methylation leads to reduction of the effective 6MP dose and decreased cytoxicity (Lennard et al., 1987). However, when tIMP is methylated, this may lead to a build up of MetIMP, which is an inhibitor of purine de novo synthesis and thereby contributes to cytotoxicity (Vogt et al., 1993). To examine whether MRP4 or MRP5 also transport MetIMP, we incubated cells with MeMPrib, which is converted to MetIMP by adenosine kinase. Uptake of MeMPrib was rapid and MetIMP was the only metabolite detected by

HPLC analysis in the intracellular and extracellular fractions (Fig. 7A). We did not detect any 6-methylated guanosines or di- or triphosphorylated methyl thiopurines. MetIMP accumulated to high concentrations in the cells (Fig. 7B) and was excreted at substantial rates (Table 3). Average MetIMP efflux rates (determined by linear regression) were 1.3, 1.9, and 3.2 pmol/10^6 cells/min for HEK293, HEK293/5I, and HEK293/4.3 cells, respectively. The relatively high rate of MetIMP secretion by the HEK293/4.3 cells is mirrored by a decreased intracellular concentration at the 4 h time point in Fig. 7B. Recovery of MeMPrib was 77 ± 23, 85 ± 15, and 79 ± 21% (mean ± S.E.M.) for the HEK293, HEK293/4.3, and HEK293/5I cells. It is possible, therefore, that we may have missed MeMPrib metabolites. The analysis of these metabolites is complicated by their relatively low absorption maximum (Table 2), which results in interference by the standard nucleotides present in cell extracts.

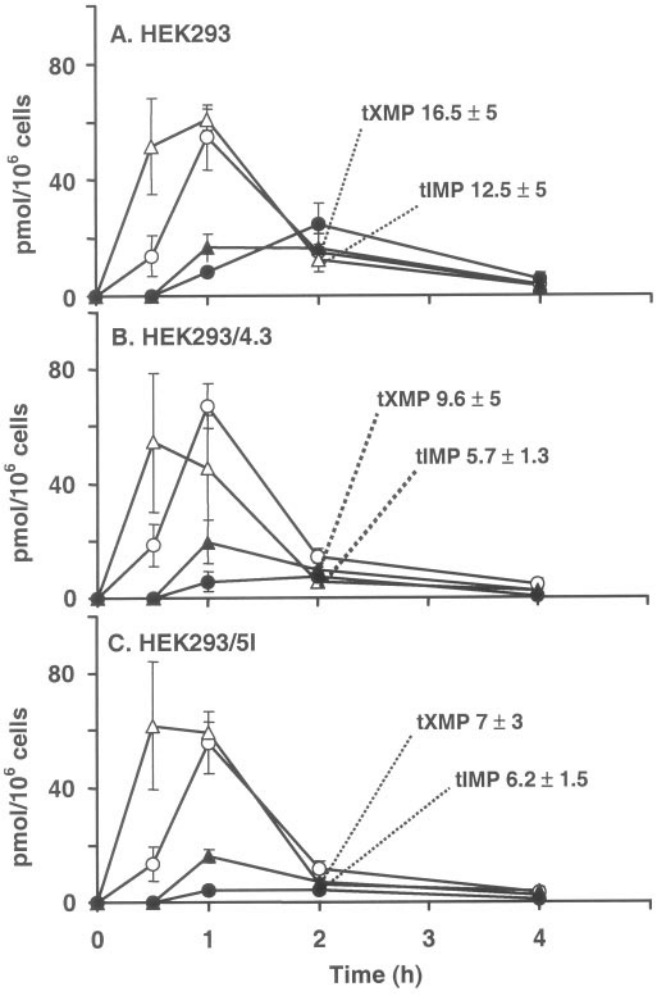

Fig. 5. Intracellular 6MP metabolites. Metabolites found in the intracellular fractions from the HEK293 (A), HEK293/4.3 (B), and HEK293/5I (C) during continuous incubation with 6MP (initial concentration, 2.5 μM). Levels of tIMP (△), MPrib (○), tXMP (▲), and TXrib (●) were quantified by relating the peak areas of detected metabolites with the peak area obtained with a 6MP and TX standards. TX levels were below the detection limit (< 1 pmol/10^6 cells). The data are the mean ± S.E.M. of three independent determinations.

Discussion

We have investigated the effect of MRP4 or MRP5 overexpression on the metabolism of thiopurines in HEK293 cells. Previous studies showed that both MRP4- and MRP5-overexpressing cells showed low-level resistance against 6MP and TG. After preloading with radiolabeled 6MP under ATP-depleting conditions, increased efflux of radiolabel was seen from the cells (Chen et al., 2001), but the analysis was hampered by low thionucleotide formation and limitations in the detection method (Wijnholds et al., 2000). Here, we circumvented these problems in two ways. First, we exposed the cells to thiopurines continuously without limiting their ATP-generating capacity; second, we adopted a reversed-phase ion-pairing HPLC system incorporating spectral UV-visible detection. The continuous exposure resulted in a more extensive, and physiologically relevant, thiopurine metabolism in the HEK293 cells, and the new detection system allowed us to more accurately identify thiopurine metabolites formed.

Under constant exposure to 6MP, the HEK293 cells exhibited extensive thiopurine metabolism. Uptake was readily saturable, and at the concentration we used routinely (2.5 μM), accumulation slowed rapidly after 1 h, reaching a maximum uptake after 4 h of 30 to 40% of the total 6MP applied. A similar pattern was observed with exposure to thioguanine. The generation of tIMP or tGMP during exposure to 6MP or TG, respectively, was the limiting step in metabolism, suggesting that the concentration of PRPP determined the amount of tNMP formed. In both cases, cells formed thiopurine nucleotides (tNMPs) and nucleosides, which were recovered both intracellularly and in the medium. The formation of all other metabolites followed the generation of the initial tNMP (see also Fig. 8). Overall, the gradual decrease in extracellular thiopurine concentration during the incubation combined with the subsequent intracellular flux through the different metabolites may approximate both clinical dosing regimens and pharmacokinetics.

All cells effluxed tNMPs when exposed to 6MP, TG, or MeMPrib, presented schematically in Fig. 8. Cells overexpressing MRP4 or MRP5 effluxed more tIMP, tGMP, and MetIMP than the parental cells; only MRP5-overexpressing cells effluxed tXMP. Because of the complex nature of the in vivo system used here, we cannot draw definitive conclusions on the relative preference of MRP4 and MRP5 for the different tNMP substrates they transport. However, based on rates calculated by simple linear regression, MRP4 seems to prefer MetIMP and tGMP, and MRP5 seems to prefer tIMP and tXMP. The affinity of MRP4 and MRP5 for tNMPs may be relatively low because our rough estimates of the intracellular concentrations of the effluxed metabolites indicate that these may reach millimolar levels. Accurate determination of the affinity of the pumps for the respective tNMPs requires vesicular uptake studies. It is also likely, in view of the substantial efflux from the parental cells, that they contain other pumps able to extrude tNMPs. Because there is a high overexpression of MRP4 or MRP5 in our transfectants, it seems unlikely that the endogenous levels of these pumps alone can account for the efflux we observe from the parental cells. MRP8 and MRP9 are possible candidates (Bera et al., 2001; Tammur et al., 2001; Yabuuchi et al., 2001).

In addition to tNMPs, the HEK293 cells also generated substantial quantities of thiopurine nucleosides upon expo-

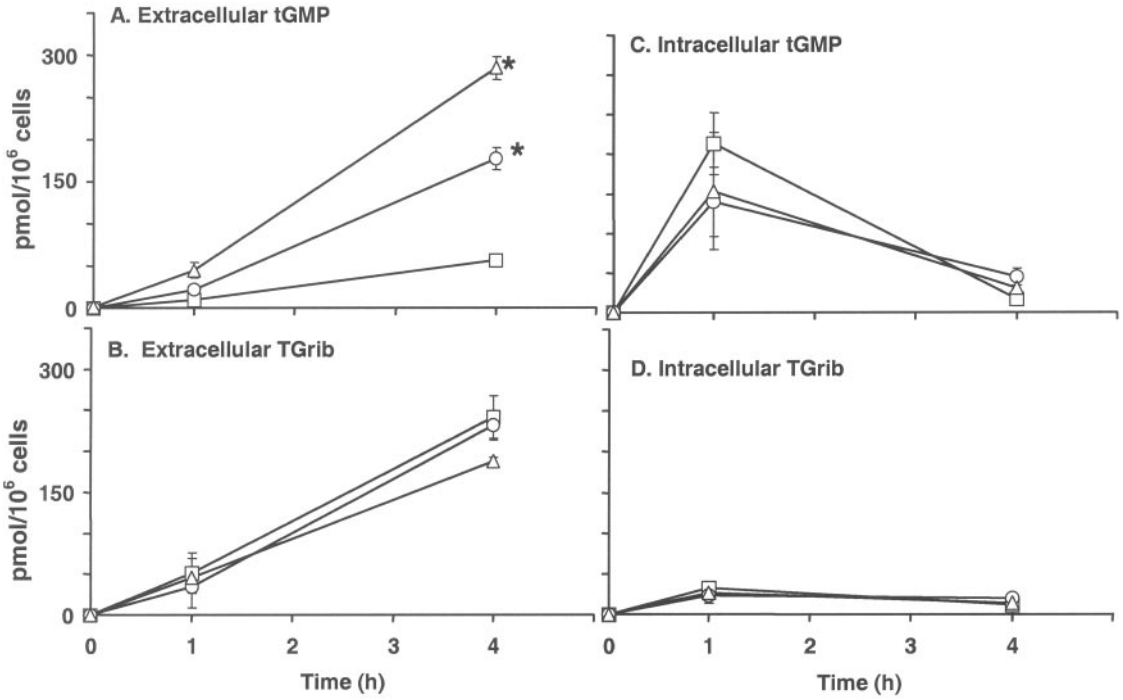

Fig. 6. Continuous incubation with TG. Thiopurine metabolites found in the extracellular (left, A and B) and intracellular (right, C and D) fractions from the HEK293 (□), HEK293/5I (○), and HEK293/4.3 (△) cells during continuous incubation with TG (initial concentration 2.5 μM). Thioguanine metabolite levels were quantified by relating the peak areas of detected metabolites with the peak area obtained with a TG standard. The data are the mean ± S.E.M. of three independent determinations. The indicated data points were significantly different (*, $p < 0.05$) from the parental cells in a Student's t test.

sure to 6MP and TG. In fact, most of the 6MP recovered extracellularly was in the form of MPrib or TXrib, and TG was found predominantly as TGrib. This is consistent with the observation that both alkaline phosphatase and 5′-nucleotidase decrease the intracellular tNMP levels (Rosman et al., 1974; Pieters et al., 1992). Once formed, the nucleosides are probably released from the HEK293 cells by equilibrative nucleoside transporters (our unpublished observations). The amount of MPrib released was equivalent for the three cell lines, whereas TGrib was slightly reduced in the HEK293/4.3 cells, presumably because of the increased tGMP efflux by these cells. Remarkably, we observed a consistent increase in the release of TXrib from HEK293 cells overexpressing MRP4. This was unexpected because TXrib is neither a nucleoside monophosphate nor an organic anion. Because thiopurine metabolism is complex and we could not use TX itself as a substrate to confirm transport, we hesitate to draw conclusions on TXrib transport by MRP4. The transport should be tested in an independent experiment (e.g., in vesicle uptake).

The anticancer activity of thiopurines is dependent on their conversion into tGMP and subsequent incorporation of tGTP into RNA and/or DNA. Any mechanism that reduces the intracellular concentration of tGMP may act as a potential resistance mechanism. As mentioned above, 5′-nucleotidase reduces the tNMP pool and has been linked to thiopurine resistance (Rosman et al., 1974; Skladanowski et al., 1996). Furthermore, TPMT methylates 6MP, forming MeMP, which reduces the 6MP concentration and toxicity (Lennard et al., 1987). Indeed, TPMT activity is inversely related to tolerance of thiopurine chemotherapy; toxicity increases in patients with low TPMT activity, whereas those with no detectable TPMT activity experience severe toxicity (Krynetski et al., 1995; Relling et al., 1999). Additionally, tIMP may be methylated, leading to a build up of MetIMP, an inhibitor of purine de novo synthesis (Vogt et al., 1993). Based on our results, it is possible that during thiopurine treatment, MRP4 and MRP5 modulate the intracellular tNMP pool via the efflux of tIMP, tXMP, tGMP, and MetIMP, and thereby contribute to cellular detoxification (Fig. 8).

In the clinic, both 6MP and TG are normally administered orally. The pharmacokinetics are relativity fast, with maximal plasma concentrations found after 2 to 3 h and a plasma half-life of 1 to 2 h (Lowe et al., 2001). MRP5 expression has been found in almost all tissues, including gut and kidneys (Kool et al., 1997; Belinsky et al., 1998), and the protein is localized to the basolateral membrane of the polarized cell line MDCKII (Wijnholds et al., 2000). MRP4 is highly expressed in the kidney (van Aubel et al., 2002) and the prostate (Lee et al., 2000) and, to a lesser extent, in the gut (unpublished observations). We find expression of both MRP4 and MRP5 in lymphoblasts from leukemia patients (unpublished observations). In light of the results presented here, MRP4 and MRP5 transporters might influence thiopurine pharmacokinetics, as well as the relative toxicity of thiopurines in both normal tissues and leukemic cells.

It is clear that MRP4 and MRP5 can transport most of the tNMPs found in patients treated with 6MP or TG. Because thiopurine nucleotide levels have been found to be predictive for treatment outcome (Lilleyman and Lennard, 1994), MRP4 and MRP5 may have a previously unrecognized involvement in thiopurine resistance and pharmacokinetics in patients. However, the potential impact of MRP4 and MRP5 will depend on the activity of other enzymes that modulate

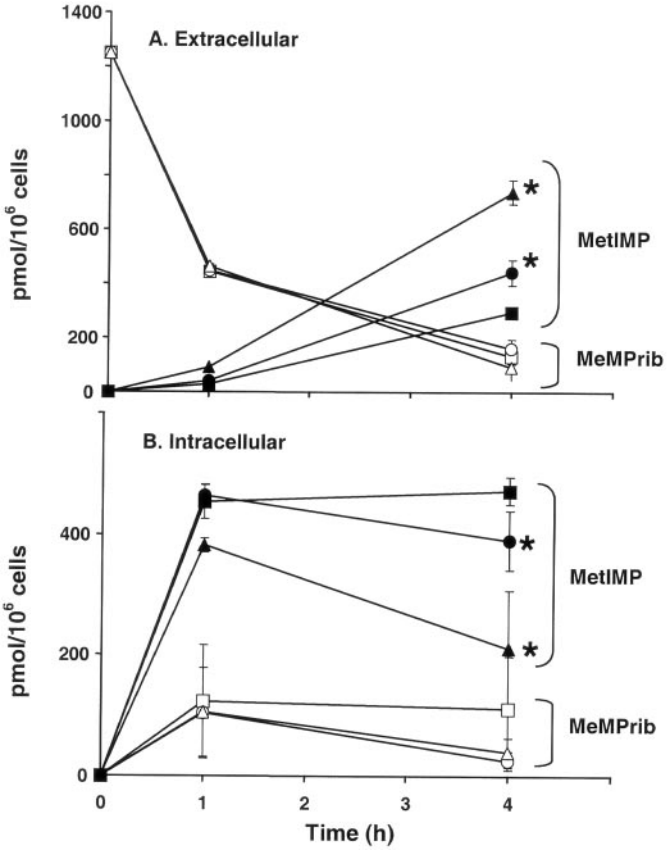

Fig. 7. Continuous incubation with MeMPrib. Thiopurine metabolites found in the extracellular (A) and intracellular (B) fractions from the HEK293 (squares), HEK293/5I (circles), and HEK293/4.3 (triangles) cells during continuous incubation with MeMPrib (initial concentration, 2.5 μM). Metabolite levels were quantified by relating the peak areas of detected metabolites with the peak area obtained with a standard amount of MeMPrib. The data are the mean ± S.E.M. of three independent determinations. The indicated data points were significantly different (*, $p < 0.05$) from the parental cells in a Student's t test.

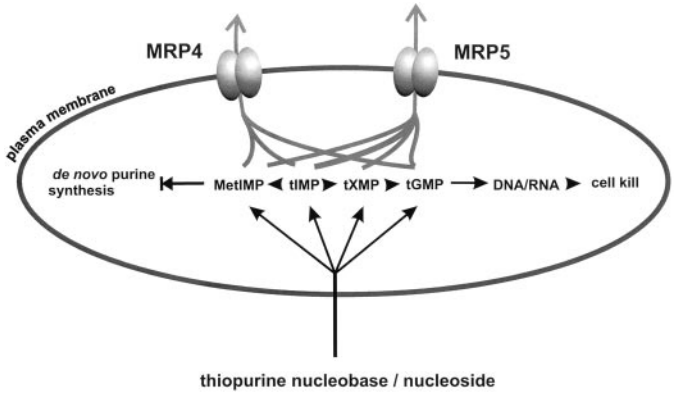

Fig. 8. Simplified scheme showing how tNMP transport by MRP4 and MRP5 might contribute to thiopurine resistance. The tNMPs transported by MRP4 and/or MRP5 are indicated. The blocked arrow indicates inhibition of de novo purine synthesis by MetIMP.

the nucleotide pool, including 5′-nucleotidase (as observed in this study) and TPMT. Most important for patients receiving 6MP treatment is the possibility that overexpression of MRP4 and MRP5 leads to a decrease in thiopurine toxicity in leukemic cells.

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