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Resistance to purine and pyrimidine nucleoside and nucleobase analogs by the human MDR1 transfected murine leukemia cell line L1210/VMDRC.06

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Abstract

Overexpression of human MDR1 P-glycoprotein [Pgp] is associated with cellular resistance to bulky amphipathic drugs, such as taxol, anthracyclines, vinca alkaloids, and epipodophyllotoxins by actively effluxing drugs from cells. We have found that human MDR1 transfected murine L1210/VMDRC.06 leukemia cells exhibit relatively large amounts of Pgp and high levels of resistance to 6-mercaptopurine [6-MP] and other purine and pyrimidine nucleobase and nucleoside analogs. L1210/VMDRC.06 cells accumulated 6-MP as the nucleotide in vitro at only about one-third of that formed by parental L1210 cells in normal medium; however, under conditions of ATP-depletion, the amount of 6-MP nucleotide formed was essentially the same in both cell lines. The findings support active efflux of 6-MP in L1210 cells, suggesting involvement of Pgp in 6-MP resistance even though it is generally believed that Pgp does not transport such agents. The resistance pattern observed in L1210/VMDRC.06 cells was not duplicated in P388/VMDRC.04 leukemia cells transfected with the same MDR1 cDNA, even though a similar amount of Pgp was present in both cell lines. Immunofluorescent staining of surface membrane Pgp showed that L1210/VMDRC.06 cells contained at least three-fold more surface Pgp than P388/VMDRC.04, implying that P388/VMDRC.04 cells are unable to actively efflux 6-MP and other antimetabolites as effectively as L1210/VMDRC.06, because of significantly lower membrane Pgp. The findings suggest that the exceedingly large concentration of overexpressed Pgp in the surface membrane of L1210/MDRC.06 cells is responsible for resistance to 6-MP and other purine and pyrimidine analogs, even though these agents usually are not considered to be substrates for Pgp.

Keywords: P-glycoprotein; Multidrug resistance; Purine and pyrimidine nucleoside and nucleobase analogs; 6-Mercaptopurine

1. Introduction

Acquired resistance of malignant cells to multiple chemotherapeutic agents, a phenomenon known as MDR, is a major obstacle to the successful treatment of cancer. Expression of plasma membrane ATP-binding cassette (ABC) transporter proteins that function as efflux pumps to actively extrude drug molecules out of the cell is one of the predominant mechanisms of MDR. Pgp, the initial drug resistance transporter discovered [1,2], has been extensively studied, both for its role in cancer drug resistance [3–7] and for its physiological functions in translocating substrates between physiological compartments [8]. The resistance spectrum of Pgp consists mainly of bulky amphipathic natural product cytotoxic drugs, such as taxanes, anthracyclines, vinca alkaloids, and epipodophyllotoxins [2–8]. Other studies have also demonstrated that Pgp is capable of transporting a large variety of physiological and synthetic molecules, including lipid analogs, cholesterol, polypeptides, and some anti-HIV drugs, such as protease inhibitors [9–12]. Thus, the substrate specificity and resistance profile of Pgp is exceedingly complex.

Following the discovery of Pgp, MRP1, was identified [13] and implicated in clinical MDR [14]. Although, MRP1 and Pgp share little sequence homology and their mechanisms of transport differ, their resistance spectra overlap to a great extent [5,6,8,13–16]. Therefore, both have been considered as targets for drug design, with the

Abbreviations: MDR, multidrug resistance; Pgp, P-glycoprotein; 6-MP, 6-mercaptopurine; MRP, multidrug resistance [associated] protein; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; PBS, phosphate buffered saline

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objective being the reversal of drug resistance through the blocking of these pumps [17–20]. In recent years, additional MRP subfamily members have been discovered and currently there are nine members, MRPs 1–9, that have been isolated and proven or suggested to be involved in drug resistance [13,14,21–25]. Among them, MRPs 4 and 5, and two new members, MRPs 8 and 9, structurally resemble Pgp, in that each contain two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs), while MRPs 1, 2, 3, 6, and 7 contain an additional third transmembrane domain at their N-termini [8,15]. This structural feature has been considered to account, at least in part, for differences in their resistance profiles and transport activities. Unlike MRPs 1, 2, and 3, which mainly mediate resistance to lipophilic anionic natural product cytotoxic drugs, and transport glutathione, glucuronate, and sulfate drug conjugates [8,14,26,27], MRPs 4 and 5, and more recently MRP 8, display moderate levels of resistance to purine analogs, such as 6-mercaptopurine [6-MP], 6-thioguanine, fluoropyrimidines and the anti-HIV drug PMEA, and transport the cyclic mononucleotides, cGMP and cAMP [28–35].

We have been studying the MDR profile of L1210/VMDRC.06 murine leukemia cells. This cell line was developed by transfection of a full-length cDNA for the human MDR1 gene [36] by Hait et al. [37] and expresses a relatively large amount of Pgp. Since, the drug resistance profile of the L1210/VMDRC.06 leukemia had not been extensively characterized, we evaluated the sensitivity of these cells to a wide variety of cytotoxic agents. We were surprised to discover that, in addition to large amphipathic natural products, L1210/VMDRC.06 cells expressed resistance to a variety of purine and pyrimidine nucleobase and nucleoside analogs.

Although, Pgp has a very limited sequence identity with MRPs 4 and 5, because of structural similarities described above, we explored the possibility that Pgp might share some of the functions of MRPs 4 and 5 in their drug resistance profile and transport activities using the MDR1 transfected murine leukemia cell line, L1210/VMDRC.06. To date, we have found that L1210/VMDRC.06 cells express resistance at a relatively high level to 6-MP (17-fold), its corresponding ribo- and deoxyribonucleosides (12- and 16-fold, respectively), 6-thioxanthine (16-fold) and 6-azauridine (21-fold). It also exhibits medium levels of resistance to 6-thioguanine (4-fold), 6-thioguanosine (4-fold), 4-thiouridine (3.7-fold), and 2′-deoxy-5-fluorouridine (8.5-fold).

The pattern of resistance to purine and pyrimidine analogs, exhibited by L1210/VMDRC.06 cells was not observed with the murine leukemia cell line, P388/VMDRC.04, which was transfected with the same *MDR1* cDNA [38], even though analyses of the full-length coding region of *MDR1* cDNA in L1210/VMDRC.06 and P388/VMDRC.04 cells were identical and confirmed the integrity of the Pgp sequence in both cell lines. This difference between these two leukemia cell lines appears to be due to the fact that L1210/VMDRC.06 cells express a 27-fold greater level of surface Pgp than parental L1210 cells; whereas, P388/VMDRC.04 cells express only an 8.3-fold greater level of surface Pgp than P388 parental cells, even though both cell lines contain similar levels of total cellular Pgp. The findings collectively support the conclusion that the relatively high concentration of Pgp expression in the plasma membrane of L1210/VMDRC.06 cells is responsible for the increased resistance of these cells to 6-MP and other purine and pyrimidine nucleoside and nucleobase analogs, presumably by the active efflux of these agents.

2. Materials and methods

2.1. Materials

6-Mercaptopurine, 6-mercaptopurine ribonucleoside, 6-mercaptopurine 2′-deoxyribonucleoside, 6-thioguanine, 6-thioguanosine, 8-azaguanine, 6-methylmercaptopurine, 6-methylmercaptopurine ribonucleoside, 6-thioxanthine, 6-azauridine, 2-thio-6-azauridine, 4-thiouridine, 4-thiouridine 5′-monophosphate, 5-fluorouracil, 5-fluorouridine, 5-fluoro-2′-deoxyuridine, 5-fluoro-5′-deoxyuridine, fludarabine, 3′-azido-3′-deoxythymidine, hypoxanthine, sodium azide, choline chloride, paclitaxel, and vinblastine were purchased from Sigma. 2′,3′-Dideoxyinosine, 2′,3′-dideoxyadenosine, 2′,3′-dideoxycytidine, 2′,3′-dideoxyguanosine, and 8-azaadenine were obtained from ICN Biomedicals. 2′,3′-Dideoxy-3′-thiacytidine and 9-(2-(phosphonomethoxy)ethyl)adenine were kindly donated by Dr. Yung-Chi Cheng (Yale University). [^{14}C]6-MP (54 mCi/mmol) was purchased from Moravek. [^{3}H]Hypoxanthine (4.5 Ci/mmol) was obtained from NEN. Monoclonal antibody C219 was supplied by Signet. FITC-conjugated murine monoclonal antibody 17F9 was obtained from Pharmingen.

2.2. Cell lines and culture conditions

L1210, L1210/VMDRC.06 [37], P388 and P388/VMDRC.04 [38] cells were generously provided by Dr. William N. Hait and Dr. Jin-Ming Yang (The New Jersey Cancer Institute, UMDNJ, New Brunswick, NJ). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 2 mmol/ml of L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Western blot analyses

Cell lysates were prepared by collecting cells (5×10^6), washing twice with phosphate buffered saline (PBS), and incubating for 2 min in lysis buffer (1% SDS,

10 mM Tris–HCl, pH 7.0, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin, 1 μg/ml of aprotinin). Lysates were filtered through a QIAshredder column (Qiagen), and filtrates were collected. Protein concentrations were measured by the Lowry method [39], using the Bio-Rad Dc protein assay. Protein samples (40 μg) were separated by 8% SDS-PAGE [40] and immunoblotted, using monoclonal antibody C219 (Signet) at a dilution of 1:500.

2.4. Cytotoxicity assay

The effects of drugs were assessed, using the Celltiter 96 aqueous one solution cell proliferation assay (Promega). Cells were exposed to various concentrations of each agent for 3 days at 37 °C and viability was assayed colorimetrically. Cytotoxicity was expressed as the IC_{50}, defined as the concentration of drug that caused 50% inhibition of growth. The degree of resistance was estimated from the ratio of the IC_{50} of L1210/VMDRC.06 cells to that of parental L1210 cells and was expressed as the fold resistance [FR].

2.5. Cellular accumulation of hypoxanthine and 6-MP

Cells (1–2 × 10^6/ml) were incubated at 37 °C with either 0.17 μM [^{3}H]hypoxanthine (14.5 Ci/mmol) or 10 μM [^{14}C]6-MP (54 mCi/mmol) in complete RPMI 1640 medium or in an ATP deficient medium [glucose-free RPMI 1640 containing 10 mM deoxyglucose and 15 mM sodium azide] plus 10% fetal bovine serum. Cells were then resuspended in the transport buffer containing 10 μM [^{14}C]6-MP at a final density of 1–2 × 10^6 cells/ml. At various times thereafter, aliquots (1.0 ml) of the cell suspension were removed and added to 10 ml of ice-cold PBS. Cells were then pelleted at 4 °C, washed twice with 10 ml each of ice-cold PBS, lysed in 1% SDS, and radioactivity therein measured by liquid scintillation spectrometry.

2.6. Hypoxanthine-guanine phosphoribosyl transferase [HGPRT] activity

HGPRT activity was measured as described by Inaba et al. [41]. Cells (2.5 × 10^7) were collected and washed twice with ice-cold PBS. Cell pellets were suspended at 4 °C in 1.5 ml lysis buffer (50 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride) for 10 min and sonicated three times for 15 s each with 30 s intervals on ice. Supernatants were collected after centrifugation of lysates at 25,000 × *g* for 30 min at 4 °C and the protein contents were determined [39]. Enzymatic activity was measured as described by others [41,42]. Briefly, 200 μl reaction mixtures were prepared containing 100 mM Tris–HCl, pH 7.4, 12 mM $MgCl_2$, 1 mM phosphoribosylpyrophosphate and 10-20 μg of protein. Mixtures were preincubated at 37 °C for 2 min, followed by the addition of 50 μl of [^{3}H]hypoxanthine (final concentration 0.6 μM, 14.5 Ci/mmol) or [^{14}C]6-MP (final concentration 0.85 mM, 54 mCi/mmol). Aliquots of 20 μl were removed every min for 10 min and added to an ice-cold EDTA solution (final concentration 0.2 M) to stop the reaction. The solutions were applied to Whatmann DE-81 filter disks, which were then dried and washed three times with 1 mM ammonium bicarbonate, once with distilled water, and once with methanol. After air-drying, radioactivity bound to disks was quantified, using a liquid scintillation spectrometer.

2.7. Measurement of 6-MP nucleotide

Cells (5 × 10^6) were incubated at 37 °C for 1 h in either complete RPMI 1640 medium or an ATP deficient medium containing 10 μM [^{14}C]6-MP (54 mCi/mmol). Cells were collected by centrifugation and washed twice with ice-cold PBS. 6-MP nucleotides were extracted twice with 2 ml of ice-cold 4% perchloric acid. Supernatants were collected and neutralized with 2N NaOH to pH 7.0, followed by centrifugation at 25,000 × *g* for 15 min at 4 °C. Supernatants were collected and aliquots were applied to Whatmann DE-81 filter disks, which were then air dried and washed three times with 1 mM ammonium bicarbonate, once with distilled water, and once with methanol. After air-drying, radioactivity in the form of nucleotides bound to the disks was quantified, using a liquid scintillation spectrometer.

2.8. RT-PCR analyses and DNA sequencing

Total RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer's protocol. Primer sequences of mouse MRPs 1–7 and β-actin used in RT-PCR studies were described previously [43]. RT-PCR was performed with 0.2 μg of total RNA in 25 μl reactions, using the Access RT-PCR System (Promega). The RT-PCR conditions were as follows: one cycle of 48 °C for 45 min and 94 °C for 2 min; 25 cycles of 94 °C for 30 s, 55 °C for 1 min, and 68 °C for 2 min, and a final extension cycle of 68 °C for 7 min. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide. The images were scanned and analyzed using a Bio-Rad gel documentation system. For DNA sequencing, total RNA isolated from L1210/VMDRC.06 and P388/VMDRC.04 cells was reverse-transcribed and amplified with human MDR1 specific primers using the high fidelity ProStar UF RT-PCR system (Stratagene). The primer sequences used were as follows: forward (5′-3′)GTCGGGATGGATCTTGAAGG, reverse (5′-3′) CAGAGTTCACTGGCGCTTTG. The amplified 3.8 kb coding region of human *MDR1* cDNA was gel-purified and sequenced. The sequence results were compared with GenBank human P-glycoprotein [Pgp] MDR1 sequence (AF016535), using the BLAST program.

2.9. Flow cytometric analysis of plasma membrane Pgp

Cells were collected and washed twice with ice-cold PBS. Cells (1×10^6) were resuspended in 100 μl of staining buffer containing 2% fetal bovine serum and 0.1% sodium azide in PBS. FITC-conjugated murine monoclonal antibody 17F9 (20 μl, Pharmingen) were added to cells and incubated on ice for 30 min. Cells were then washed with staining buffer twice to remove unbound antibody and then resuspended in 500 μl of staining buffer for flow cytometric analysis. Relative fluorescence intensities were obtained by comparing the fluorescence intensity of Pgp-overexpressing cells to that of parental cells.

3. Results

The expression levels of human Pgp in wild-type and MDR1 transfected L1210 and P388 cells were determined by western analysis using monoclonal antibody C219. Pgp was present in a similar relatively high concentration in both L1210/VMDRC.06 and P388/VMDRC.04 transfected cells, while little or no Pgp was detected in their corresponding parental cells (Fig. 1). The MDR1 transfected cell lines, established by Dr. William N. Hait and Dr. Jin-Ming Yang, were shown to exhibit a drug resistance profile expected for Pgp [37,38]. The functional activity of Pgp in these cell lines was verified by our laboratory, using an MTS/PMS cytotoxicity assay; a relatively large increase in the resistance of MDR1 transfected cells to paclitaxel (10- to 40-fold) and vinblastine (10-fold) over that of parental cells was observed, both of which could be reversed by 20 μM verapamil.

In studies being conducted with 6-MP, we discovered that, while parental L1210 cells were sensitive to this agent, with an IC50 value of 0.23 μM, L1210/VMDRC.06 cells were 17.2-fold more resistant, with an IC50 value of 4.0 μM (Table 1, Fig. 2A). For this reason, we determined the sensitivity of L1210/VMDRC.06 cells to a variety of purine and pyrimidine nucleobase and nucleoside analogs using the standard Celltiter 96 aqueous one solution cell proliferation assay. In addition to resistance to 6-MP, L1210/VMDRC.06 cells exhibited a relatively high level of resistance to 6-MP ribonucleoside (16.4-fold), 6-MP 2′-deoxyribonucleoside (11.5-fold), 6-azauridine (21.2-fold), and 2-thio-6-azauridine (11.8-fold) (Table 1, Fig. 2). L1210/VMDRC.06 cells also showed an intermediate level of resistance to 6-thioguanine, 6-thioguanosine and 4-thiouridine (4-fold), and a low level of resistance to 8-azaguanine (2.4-fold). Interestingly, a significant degree of resistance to 5-fluoro-2′-deoxyuridine (8.5-fold) was observed for L1210/VMDRC.06 cells, but no resistance to 5-fluorouracil or 5-fluorouridine (Fig. 3). As summarized in Table 1, L1210/VMDRC.06 cells exhibited increased resistance to several additional nucleobase ana-

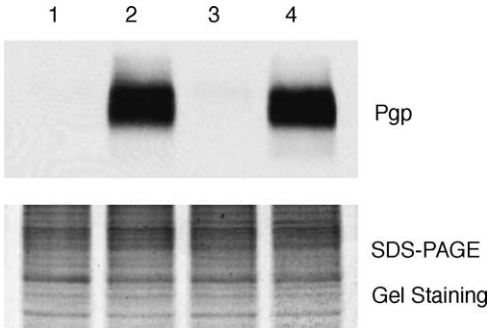

Fig. 1. Western analysis of MDR1 transfected L1210 and P388 cells. Forty micrograms of total protein from L1210/VMDRC.06 cells or P388/VMDRC.04 cells were separated by 8% SDS-PAGE. Pgp was detected using murine C219 monoclonal antibody. Lane 1, L1210 cells; Lane 2, L1210/VMDRC.06 cells; Lane 3, P388 cells; Lane 4, P388/VMDRC.04 cells. The bottom panel is a section of an identical gel stained with Simply Blue solution [Invitrogen] to demonstrate approximately equal loading.

Table 1
Drug sensitivity of L1210 and L1210/VMDRC.06 cells

Drug[a]	IC50 [μM][b]		N[c]	FR [fold resistance][d]
	L1210	L1210/VMDRC.06		
6-MP[e]	0.23 ± 0.05	4.0 ± 2.0	9	17.2
6-MP 2′-deoxyR	0.33 ± 0.11	3.8 ± 1.4	7	11.5
6-MPR	0.22 ± 0.08	3.6 ± 2.2	5	16.4
6-Thioguanine	0.19 ± 0.04	0.78 ± 0.21	9	4.1
6-Thioguanosine	0.23 ± 0.07	0.89 ± 0.12	7	3.9
8-Azaguanine	0.18 ± 0.04	0.44 ± 0.09	7	2.4
6-Methyl-MP	662 ± 55	1400 ± 210	6	2.1
6-Methyl-MPR	0.26 ± 0.15	0.37 ± 0.15	6	1.4
6-Thioxanthine	101 ± 41	1620 ± 820	7	16.0
6-Azauridine	0.79 ± 0.57	16.7 ± 8.1	6	21.2
2-Thio-6-AzaU	3.1 ± 2.9	36.8 ± 26.8	8	11.8
4-Thiouridine	8.0 ± 3.4	29.8 ± 12.6	7	3.7
4-ThioU 5′-monoP	6.4 ± 3.6	24.2 ± 6.9	5	3.8
5-Fluoro-2′-deoxyU	0.003 ± 0.002	0.022 ± 0.012	6	8.5
5-Fluorouridine	0.014 ± 0.013	0.012 ± 0.007	3	0.84[f]
5-Fluorouracil	0.62 ± 0.69	0.62 ± 0.69	3	1.0[f]
5-Fluoro-5′-deoxyU	9.6 ± 5.6	8.5 ± 2.1	3	0.89[f]
Fludarabine	8.0 ± 2.8	5.0 ± 2.8	3	0.63[f]
3TC	2460 ± 820	6000 ± 1000	3	2.4
PMEA	45 ± 13	73 ± 11	3	1.6
AZT	3160 ± 300	4540 ± 840	5	1.4

[a] No resistance detected for 2′,3′-dideoxyinosine, 2′,3′-dideoxyadenosine, 2′,3′- dideoxycytidine, 2′,3′-dideoxyguanosine, and 8-azaadenine.

[b] Drug concentration that inhibited cell survival by 50%.

[c] Number of independent experiments, each performed in quadruplicate.

[d] FR [fold resistance]: IC_{50} of L1210/VMDRC.06 cells/IC_{50} of L1210 cells.

[e] *Abbreviations:* 6-MP, 6-mercaptopurine; 6-MP 2′-deoxyR, 6-mercaptopurine 2′-deoxyribonucleoside; 6-MPR, 6-mercaptopurine ribonucleoside; 6-methyl-MP, 6-methyl-mercaptopurine; 6-methyl-MPR, 6-methylmercaptopurine ribonucleoside; 2-thio-6-azaU, 2-thio-6-azauridine; 4-thioU 5′-monoP, 4-thiouridine 5′-monophosphate; 5-fluoro-2′-deoxyU, 5-fluoro-2′-deoxyuridine; 5-fluoro-5′-deoxyU, 5-fluoro-5′-deoxyuridine; 3TC, 2′,3′-dideoxy-3′-thiacytidine; PMEA, 9-[2-[phosphonomethoxy]ethyl]adenine; AZT, 3′-azido-3′-deoxythymidine.

[f] Not significantly different [$P > 0.05$] as assessed by Student's *t*-test. The rest of the values in the Table are significantly different [$P < 0.001$] as assessed by Student's *t*-test.

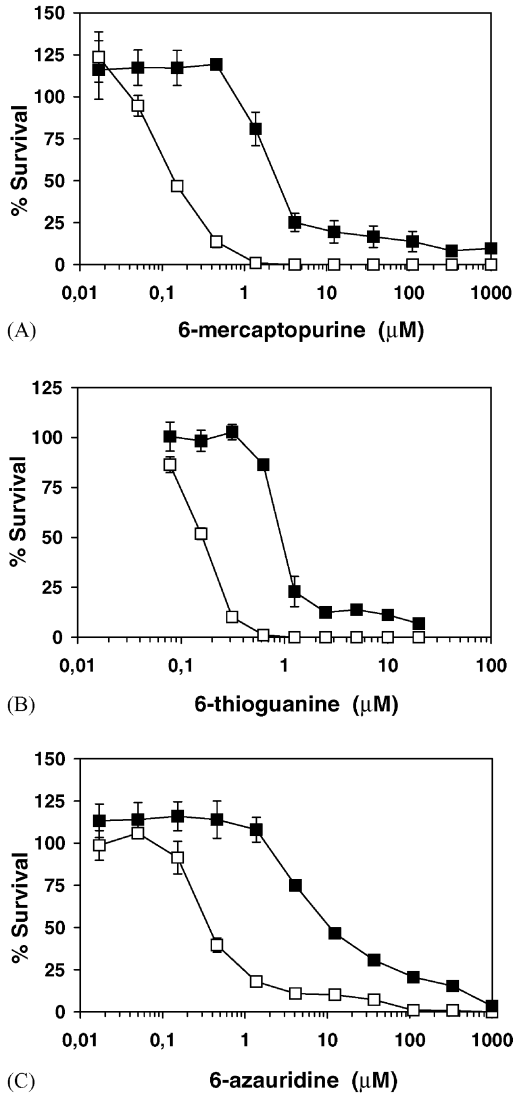

Fig. 2. Sensitivity of L1210/VMDRC.06 cells to 6-mercaptopurine [A], 6-thioguanine [B], and 6-azauridine [C]. The drug sensitivity of parental L1210 cells (□) and L1210/VMDRC.06 cells (■) was analyzed, using the Celltiter 96 aqueous one solution cell proliferation assay as described in Section 2. Data points represent the mean ± S.D. of quadruplicate determinations in a representative experiment.

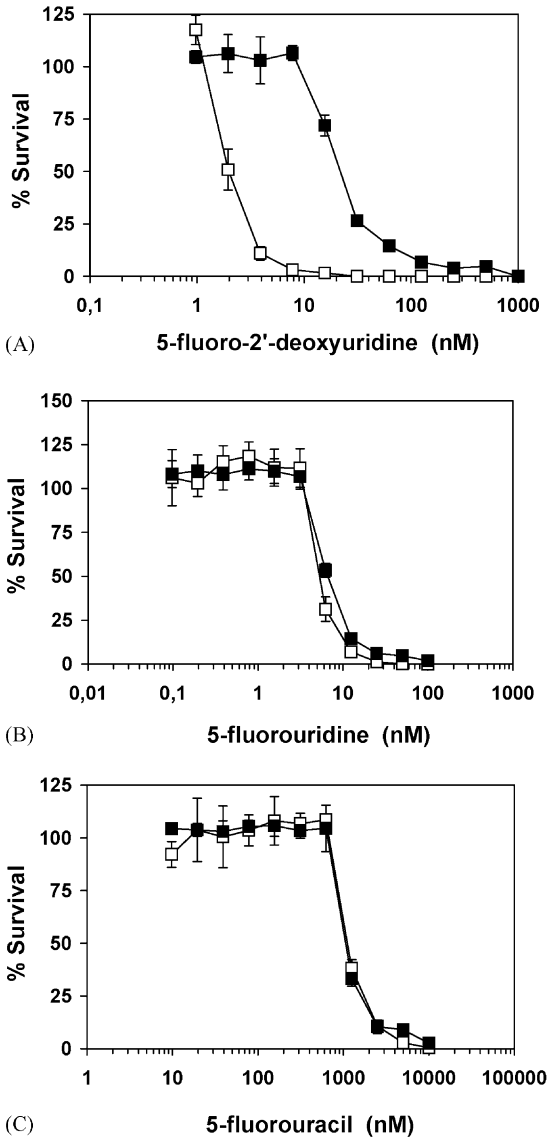

Fig. 3. Sensitivity of L1210/VMDRC.06 cells to 5-fluoro-2′-deoxyuridine [A], 5-fluoro- uridine [B], and 5-fluorouracil [C]. The drug sensitivity of parental L1210 cells (□) and L1210/VMDRC.06 cells (■) was analyzed, using the Celltiter 96 aqueous one solution cell proliferation assay as described in Section 2. Data points represent the mean ± S.D. of quadruplicate determinations in a representative experiment.

logs, and a few antiviral agents, albeit at relatively low levels. In contrast, L1210/VMDRC.06 cells did not show resistance to fludarabine, and a few other nucleoside analogs, including 2′,3′-dideoxyinosine, 2′,3′-dideoxyadenosine, 2′,3′-dideoxycytidine, 2′,3′-dideoxyguanosine, and 8-azaadenine (data not shown). Probenecid decreased the degree of resistance of L1210/VMDRC.06 cells to both 1 μM 6-MP and 10 μM paclitaxel by about 50%, while verapamil and cyclosporine A did not affect the degree of resistance to the 6-thiopurine (unpublished observations); it is known that inhibitors of this transporter can have different mechanisms of action at the level of Pgp.

Of interest, was the finding that the pattern of resistance to these agents exhibited by L1210/VMDRC.06 cells was not observed with the closely related P388/VMDRC.04 cells, even though both cell lines were transfected with the same MDR1 vector and expressed a similar high amount of MDR1. In contrast to L1210/VMDRC.06, P388/VMDRC.04 cells displayed minimal resistance to 6-azauridine (2-fold) and 6-thioguanine (1.7-fold), and no resistance to 5-fluoro-2′-deoxyuridine, compared to parental P388 cells (Fig. 4); because of the intrinsic resistance of parental P388 cells to 6-MP, high enough concentrations of this agent were not reached to generate meaningful kill curves (data not shown).

Since, efflux by the Pgp would appear to be the only reasonable mechanism by which such a diverse spectrum of resistance to purine and pyrimidine nucleobase and nucleoside analogs could occur in L1210/VMDRC.06

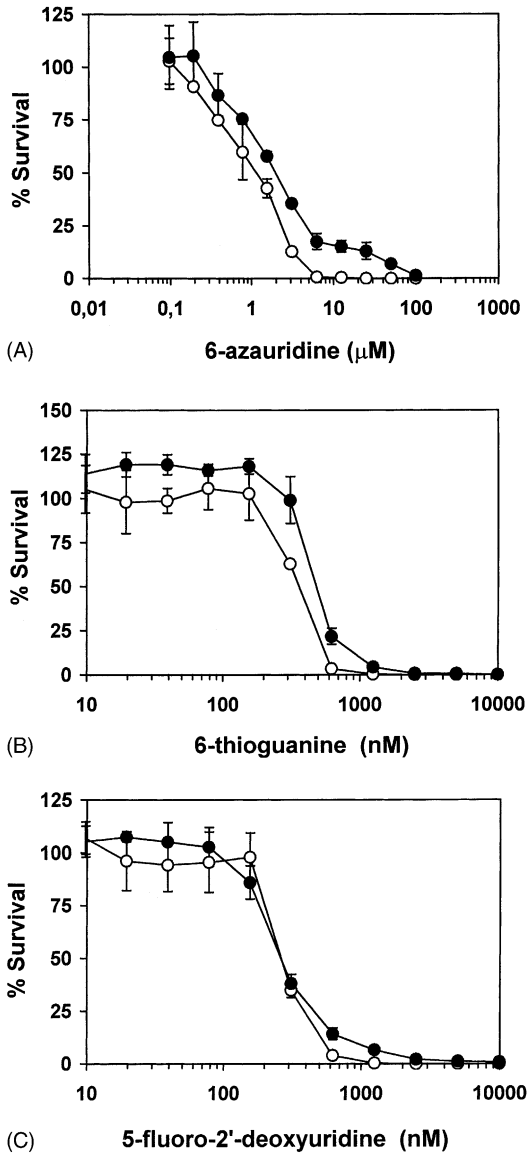

Fig. 4. Sensitivity of P388/VMDRC.04 cells to 6-azauridine [A], 6-thioguanine [B], and 5-fluoro-2′-deoxyuridine [C]. The drug sensitivity of parental P388 cells (○) and P388/VMDRC.04 cells (●) was analyzed, using the Celltiter 96 aqueous one solution cell proliferation assay as described in Section 2. Data points represent the mean ± S.D. of quadruplicate determinations in a representative experiment.

cells, we have conducted studies with 6-MP to gain insight into whether such a mechanism was operative. Initial experiments were conducted to compare the cellular influx of radiolabeled 6-MP and hypoxanthine (as a model purine) by L1210/VMDRC.06 MDR1 transfected cells and parental L1210 cells. Accumulation of [^{3}H]hypoxanthine was significantly lower in L1210/VMDRC.06 cells than in parental L1210 cells (Fig. 5A) and a similar accumulation deficit was also noted for [^{14}C]6-MP, with the number of 6-MP molecules accumulated by L1210/VMDRC.06 cells being only about one-half of that occurring in parental L1210 cells after 1-h of incubation with 10 μM [^{14}C]6-MP (Fig. 5B). The accumulation difference for [^{14}C]6-MP between L1210 and L1210/VMDRC.06 cells disappeared under conditions of energy depletion using 15 mM sodium azide and replacement of glucose by 2′-deoxyglucose, with uptake by energy depleted L1210/VMDRC.06 cells being identical to that of parental L1210 cells (Fig. 5C). Furthermore, the finding that virtually no [^{14}C]6-MP was influxed in either L1210 or L1210/VMDRC.06 cells on ice (4 °C) implies that 6-MP enters cells by a passive process involving a structural region of the membrane for uptake (Fig. 5D).

Since, a decrease in the activity of HGPRT is a major known mechanism of resistance to the 6-thiopurine analogs, we measured HGPRT activity in L1210 and L1210/VMDRC.06 cellular extracts. As shown in Table 2, the initial rates of nucleotide formation were not significantly different between enzyme preparations from L1210 and L1210/VMDRC.06 cells, using either hypoxanthine or 6-MP as substrates, which indicates that the 6-MP nucleotide forming system is intact in L1210/VMDRC.06 cells. In support of this notion, measurement of 6-MP nucleotide formation in intact L1210 and L1210/VMDRC.06 cells revealed that L1210/VMDRC.06 cells contained considerably less 6-MP nucleotide than L1210 cells in complete medium (Fig. 6A), while under conditions of ATP-depletion, the level of 6-MP nucleotide was equivalent in both lines (Fig. 6B). This finding demonstrated that the nucleotide forming machinery of L1210/VMDRC.06 cells is not impaired and that the influx of 6-MP is not altered in L1210/VMDRC.06 cells, but instead, that drug efflux, presumably by Pgp, accounts for the decreased intracellular levels of this agent.

To eliminate the possibility that differences in the expression of MRPs 4 and 5, two known transporters capable of conferring resistance to 6-MP and 6-thioguanine, might be involved, the expression levels of these transporters, as well as of other characterized MRP family members, were measured by semi-quantative RT-PRC. No major differences were observed for MRPs 1–7 in L1210 and L1210/VMDRC.06 cells (Fig. 7).

The observed difference in resistance to 6-MP (data for P388 and P388/VMDRC.04 not shown) and to 6-azauridine, 6-thioguanine and 5-fluoro-2′-deoxyuridine (Figure 2, 3, and 4) between MDR1 transfected L1210/VMDRC.06 and P388/VMDRC.04 cells implied that even though both cell lines contained similar quantities of total Pgp, another property was involved in the difference between L1210/VMDRC.06 and P388/VMDRC.04. To gain insight into possible differences, we examined the integrity of the full length coding sequence of the *MDR1* cDNA in both L1210/VMDRC.06 and P388/VMDRC.04 cells by RT-PCR. Through this approach we confirmed that the sequence of the coding region of the *MDR1* gene in both cell lines was identical, being devoid of mutations and/or truncations and matching well with published data.

It has been recently reported that the mislocalization of the membrane protein MRP1, presumably caused by a partly or unglycosylated form accumulating in the cytoplasm, was associated with reduced cisplatin accumulation

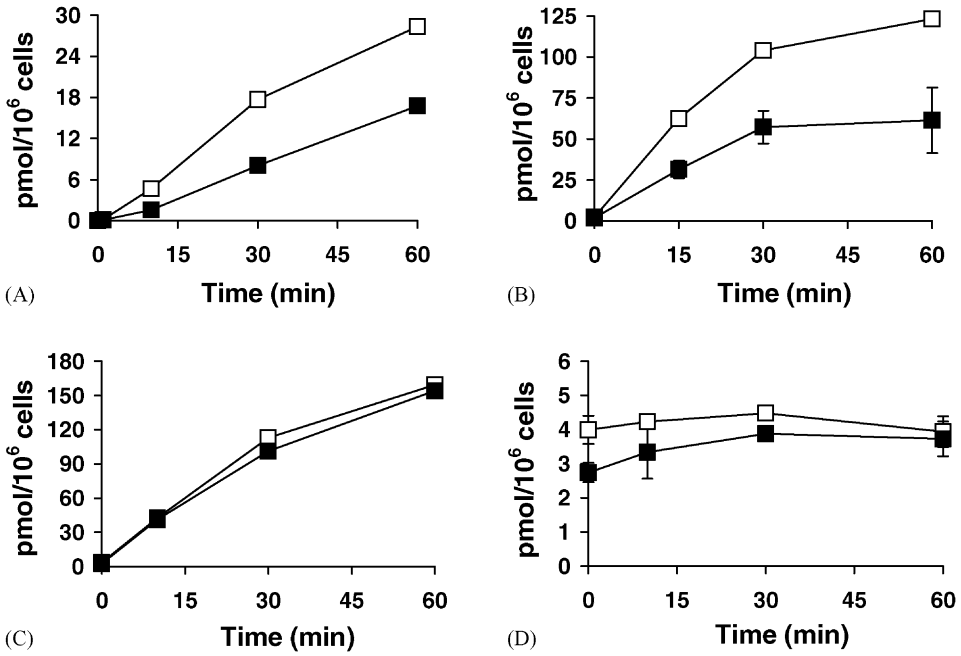

Fig. 5. Accumulation of radiolabeled hypoxanthine and 6-mercaptopurine in L1210 and L1210/VMDRC.06 cells. Parental L1210 cells (□) and L1210/VMDRC.06 cells (■) were incubated in either 0.17 μM [^{3}H]hypoxanthine in complete RPMI 1640 medium at 37 °C [A], 10 μM [^{14}C]6-mercaptopurine in complete RPMI 1640 medium at 37 °C [B], 10 μM [^{14}C]6-mercaptopurine in ATP deficient medium [glucose-free RPMI 1640 containing 10% fetal bovine serum, 10 mM deoxyglucose and 15 mM sodium azide] at 37 °C [C], or 10 μM [^{14}C]6-mercaptopurine in complete RPMI 1640 medium on ice [D]. Drug accumulation was measured at various time points as described in Section 2. Data points represent the mean of duplicate determinations in a representative experiment. Each experiment was repeated at least three times with similar results.

in cisplatin-resistant variants of human KB adenocarcinoma cells [44]. Therefore, we determined whether differences occurred in the membrane localization of Pgp between L1210/VMDRC.06 and P388/VMDRC.04 cells, being cognizant of the fact that both of these cell lines exhibited similar levels of total Pgp (Fig. 1). To quantitatively measure Pgp levels on the plasma membrane surface, unfixed, viable L1210, L1210/VMDRC.06, P388 and P388/VMDRC.04 cells were incubated with the FITC-conjugated monoclonal antibody 17F9, which binds specifically to the surface epitope of Pgp. The immunofluorescent staining of cell

Table 2
HGPRT activity of L1210 and L1210/VMDRC.06 cells using hypoxanthine and 6-mercaptopurine as substrates

Cell lines	Hypoxanthine [pmol/mg/min]	6-MP [nmol/mg/min]
L1210	0.29 ± 0.09	1.20 ± 0.48
L1210/VMDRC.06	0.30 ± 0.09	1.02 ± 0.44

Enzymatic activity was measured as described in Section 2. Protein extracts (10–20 μg) from L1210 and L1210/VMDRC.06 cells were incubated in 200 μl of reaction mixture containing 100 mM Tris–HCl, pH 7.4, 12 mM $MgCl_2$, 1 mM phosphoribosyl pyrophosphate at 37 °C for 2 min, followed by the addition of 50 μl of 3 μM [^{3}H]hypoxanthine or 4.25 mM [^{14}C]6-MP. Aliquots of 20 μl were removed every min for 10 min, and added to 5 μl of 1 M ice-cold EDTA to stop the reaction. The solutions were applied to Whatmann DE-81 filter disks, which were then dried and washed three times with 1 mM ammonium bicarbonate, once with distilled water, and once with methanol. After air-drying, radioactivity bound to disks was measured, using a liquid scintillation spectrometer.

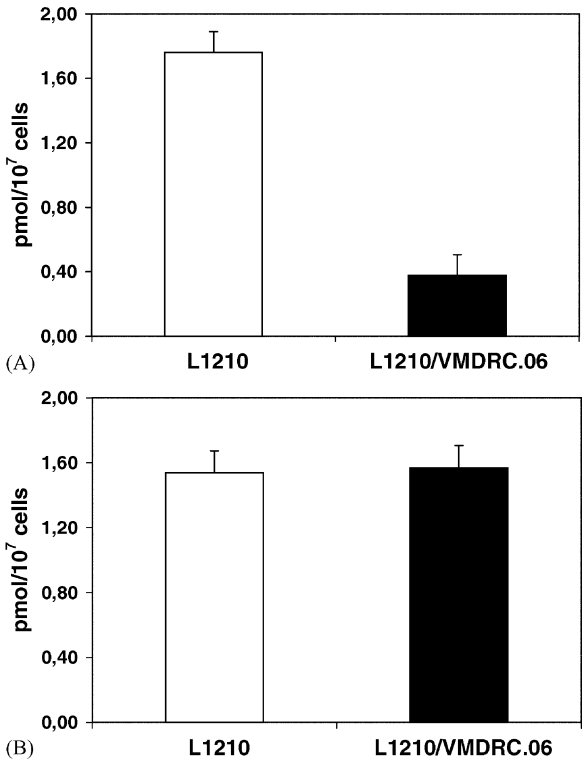

Fig. 6. Formation of 6-mercaptopurine nucleotide in L1210 and L1210/VMDRC.06 cells. Parental L1210 cells or L1210/VMDRC.06 cells were incubated in 10 μM [^{14}C]6-mercaptopurine in either complete RPMI 1640 medium [A] or ATP deficient medium [B] for 1 h. 6-MP nucleotide was extracted and quantified as described in Section 2. Data points represent the mean ± S.D. of three independent experiments.

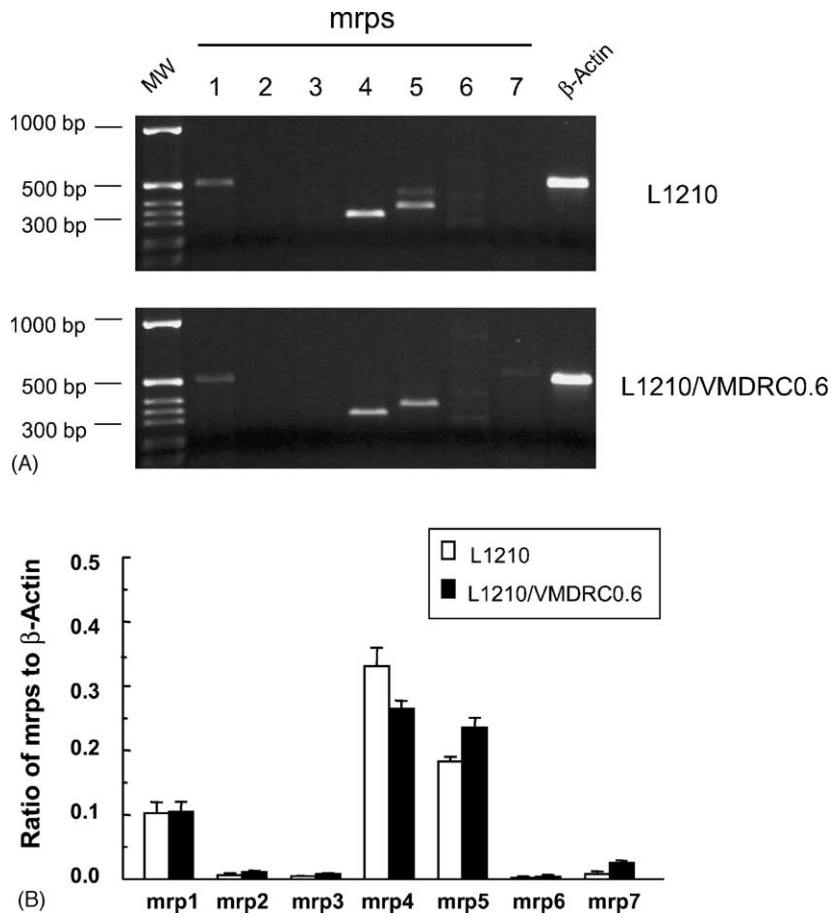

Fig. 7. RT-PRC analysis of the expression of MRPs 1–7 in L1210 and L1210/VMDRC.06 cells. Total RNA [0.2 μg] from cells was reverse-transcribed and amplified individually using primers specific for mouse mrp1, 2, 3, 4, 5, 6, 7, and β-actin. The PCR products were visualized on an agarose gel stained with ethidium bromide. Representative results are shown [A]. Band intensities of the PCR products of MRPs 1–7 were quantified and normalized to that of β-actin [B]. Values are the mean ± S.D. of three independent experiments.

surface Pgp was then analyzed by flow cytometry. L1210/VMDRC.06 cells exhibited a 27-fold greater level of surface Pgp than L1210 parental cells, as determined by relative fluorescence intensity (Fig. 8A), while in contrast, P388/VMDRC.04 cells displayed only an 8.3-fold greater level of surface Pgp than P388 parental cells (Fig. 8B). This finding demonstrated that L1210/VMDRC.06 cells contain a substantially higher level of surface membrane Pgp than P388/VMDRC.04 cells.

4. Discussion

The surprising finding that L1210 leukemia cells transfected with the human *MDR1* gene exhibit resistance to the 6-thiopurines, 6-azauridine and 5-fluoro-2′-deoxyuridine prompted a study of the possibility that resistance was the result of the cellular efflux of these agents by Pgp. This possibility was considered simply because of structural similarities between Pgp and MRPs 4 and 5, coupled with the role of MRPs 4 and 5 in purine analog resistance [28–30,33]. Several investigations have suggested that MDR1 may be involved in resistance to purine analogs using drug selected cell lines [45,46], mRNA differential display [47], and AML patient samples [48]; however, no direct evidence is available to support this possibility, which is inconsistent with the generalization that Pgp transports relatively large amphipathic natural product molecules. The involvement of Pgp in the phenomenon of resistance of L1210/VMDRC.06 cells to a number of thio-substituted purine and pyrimidine nucleobase and nucleoside analogs may be, in part, the result of increased lipophilicity imparted by the thio-substituent; however, the pronounced resistance of these cells to 6-azauridine and 5-fluoro-2′-deoxyuridine, in the absence of insensitivity to 5-fluorouracil and 5-fluorouridine, is unexplained, although it is conceivable that Pgp has the capacity to distinguish between these fluorinated pyrimidines.

We have chosen to study the involvement of Pgp in the resistance of L1210/VMDRC.06 cells to 6-MP, since a great deal is known about mechanisms of resistance to this agent [49,50]. In order for purine and pyrimidine nucleobase and nucleoside analogs to exert cytotoxicity they must be first converted to the nucleotide level [49,50]. Since, reduced HGPRT activity has been a major established mechanism of resistance to the 6-thiopurine analogs in

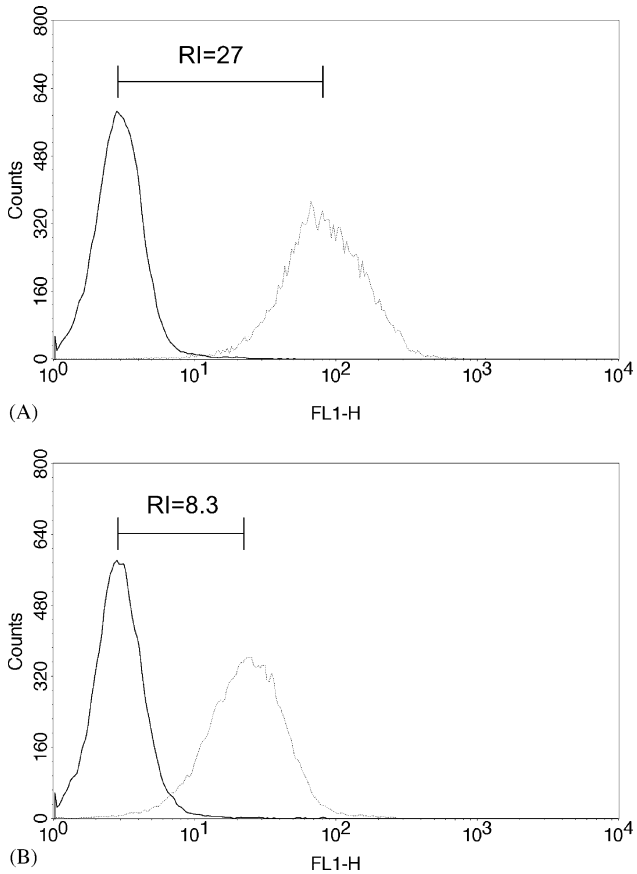

Fig. 8. Flow cytometric analysis of surface Pgp expression in L1210 and L1210/VMDRC.06 cells [A], and P388 and P388/VMDRC.04 cells [B]. Viable cells were stained with FITC-conjugated murine monoclonal 17F9 antibody and analyzed by flow cytometry. Bold line, parental cells; Thin line, MDR1 transfected cells. Relative fluorescence intensity [RI] is shown.

experimental systems [49], we explored this possibility using both enzyme preparations from L1210 and L1210/VMDRC.06 cells, as well as intact sensitive and resistant cells to demonstrate that the 6-MP nucleotide forming system was intact in L1210/VMDRC.06 cells, thereby eliminating deletion of HGPRT as a mechanism of resistance to the 6-thiopurines.

The level of resistance usually corresponds to the degree of drug accumulation for the taxanes, vinca alkaloids, epipodophillotoxins, and anthracyclines; however, this was not the case for 6-MP, in that L1210/VMDRC.06 cells exhibited a 17.2-fold increase in resistance to the 6-thiopurine relative to its L1210 counterpart, with only a 3-fold decrease in drug accumulation. The 6-thiopurines are anabolized to a variety of nucleotide forms and are incorporated into RNA and DNA in processes required for cytotoxicity; the form(s) of 6-MP effluxed by Pgp is unknown. Thus, it is conceivable that the most cytotoxic nucleotide form(s) of this 6-thiopurine is preferentially effluxed by the resistant cells, while minor amounts of less cytotoxic forms of 6-MP are retained by the cells. In partial support of this concept, a pronounced decrease in 6-MP nucleotide occurs in these cells (Fig. 6A). Importantly, the accumulation deficit of 6-MP disappeared under conditions of ATP-depletion, supporting the involvement of an ATP-dependent efflux mechanism rather than of an active influx pump, since the level of 6-MP nucleotides in L1210/VMDRC.06 cells under conditions of ATP depletion increased to that present in parental cells. Moreover, the observation that the influx of 6-MP into cells did not occur at 4 °C indicated that 6-MP enters cells by a passive process in which the rate-limiting barrier for permeation is a structural region of the membrane. These findings collectively strongly support the increased efflux of 6-MP and/or its nucleoside/nucleotide forms by an ATP-dependent transporter, presumably Pgp, in L1210/VMDRC.06 cells as the cause of resistance. The presence of resistance to the uridine analog 6-azauridine by L1210/VMDRC.06 cells, and even more interestingly, the difference in specificity to the fluorinated pyrimidines exhibited by L1210/VMDRC.06 cells, with resistance to 5-fluoro-2′-deoxyuridine coupled with retention of sensitivity to 5-fluorouracil and 5-fluorouridine, indicate some unusual specificities of Pgp in L1210/VMDRC.06 cells, assuming this transporter is also involved in the efflux of these compounds. The fact that the unusual finding of 6-MP and/or its nucleoside or nucleotide forms being effluxed by Pgp has not been previously documented is probably due to this drug and related agents normally not serving as substrates for Pgp; thus, the observed resistance and efflux of 6-MP in L1210/VMDRC.06 cells is possibly due to the exceedingly high expression of this transporter in the surface membrane of transfected cells and/or the micro-environment of the surface membrane of these cells influencing the spectrum of agents transported. This latter possibility is supported by the recent report of Gayer et al. [51] that the intracellular retention of daunomycin increased in a linear manner with the level of depletion of cholesterol in multidrug resistant neoplastic cells, indicating that the resistant phenotype exhibited by Pgp depends upon the lipid composition of the membrane. Therefore, Pgp may have a much wider spectrum of substrates heretofore appreciated. Although increased expression of Pgp has been documented in the human leukemias (see Ref. [52] for relevant references), the clinical relevance of the finding of the transport of 6-MP by the Pgp in L1210/VMDRC.06 cells is unknown.

P388 leukemia cells appeared to be inherently more resistant to 6-MP, 6-thioguanine, 6-azauridine, and 5-fluoro-2′-deoxyuridine than L1210 cells. Although, the mechanism involved in the differential sensitivity of these two murine leukemia cell lines to these agents is unknown, it was our expectation that P388 leukemia cells transfected with the MDR1 vector would express a further increase in the level of resistance to these agents if they were also pumped by the Pgp, particularly, since the total amount of this transporter in P388/VMDRC.04 cells is comparable to that present in L1210/VMDRC.06 cells and the full length coding sequence of the MDR1 gene in both cell lines is identical [data not shown]. However, this was not the case.

The fact that P388/VMDRC.04 cells had about one-third of the total cellular Pgp in the surface membrane compared to that of L1210/VMDRC.06 cells provides a possible explanation, in that the total capacity of Pgp in P388/VMDRC.04 cells to accomplish the efflux of 6-MP would be markedly diminished. This mechanism corresponds to the finding of Liang et al. [44] that mislocalization of MRP1 to the cytoplasm was associated with a reduced accumulation of cisplatin in cisplatin-resistant variants of KB adenocarcinoma cells.

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