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Antifolate Resistance Associated with Loss of MRP1 Expression and Function in Chinese Hamster Ovary Cells with Markedly Impaired Export of Folate and Cholate

MICHAL STARK, LILAH ROTHEM, GERRIT JANSEN, GEORGE L. SCHEFFER, I. DAVID GOLDMAN, and YEHUDA G. ASSARAF

Department of Biology, the Technion, Haifa, Israel (M.S., L.R., Y.G.A.); Departments of Rheumatology (G.J.) and Pathology (G.L.S.), VU Medical Center, Amsterdam, the Netherlands; and Departments of Medicine and Molecular Pharmacology, Albert Einstein Cancer Center, Albert Einstein College of Medicine, Bronx, New York (I.D.G.)

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ABSTRACT

Export of folates from a Chinese hamster ovary Pyr^{R100} cell line is markedly impaired, resulting in expansion of cellular folate pools and high-level antifolate resistance. We now report that MRP1 expression is absent in Pyr^{R100} cells along with a marked decrease in MRP5 expression with 3-fold cross-resistance to thiopurines. Pyr^{R100} and wild-type cells had comparable low levels of MRP2 expression; both lacked the breast cancer resistance protein. Pyr^{R100} cells showed a 4-fold decrease in cholate (an MRP substrate) efflux with a 6-fold increase in cellular cholate accumulation compared with wild-type cells. Prostaglandin A_1 increased cholate accumulation in wild-type cells to levels comparable with Pyr^{R100} cells. Calcein (an MRP1 substrate) fluorescence increased 5-fold in Pyr^{R100} cells; probenecid increased the intracellular calcein level in wild-type cells to that of Pyr^{R100} cells. Consistent with the loss of MRP1 expression, Pyr^{R100} cells showed modest collateral sensitivity to cholate, etoposide, doxorubicin, and vincristine. Transfection of MRP5 into Pyr^{R100} cells did not alter sensitivity to pyrimethamine or MTX but restored sensitivity to mercaptopurines, indicating that decreased MRP5 expression did not play a role in antifolate resistance. Hence, although MRP-mediated anticancer drug resistance has been associated with *gain* of function (i.e., overexpression), this is the first report that *loss* of MRP1 efflux function can expand intracellular folate pools to result in acquired antifolate resistance. The data also suggest that MRP1, and possibly other MRPs that transport folates, can play a role in the maintenance of cellular folate homeostasis.

Mammalian cells lack the biochemical pathways necessary for endogenous folate synthesis and hence require specific processes that mediate the delivery of exogenous hydrophilic folate molecules into cells. This is accomplished by a variety of uni- and bidirectional transporters recently reviewed in detail (Matherly and Goldman, 2003). The reduced folate carrier (RFC; SLC19A1) is a bidirectional facilitative system that generates transmembrane folate gradients by exchange with organic phosphates concentrated within cells. Folate receptors mediate the unidirectional transport of folates into mammalian cells by a GPI-linked, energy-requiring endocytotic mechanism. There are, in addition, several organic anion facilitative carriers (the SLC21 family) expressed in epithelial cells and some tumors that transport folates and antifolates (Hagenbuch and Meier, 2003). These processes are opposed by at least five ATP-driven, unidirectional ABC exporters, including MRP1–4 and the breast cancer resistance protein (BCRP) that pump folates out of cells (Borst and Oude Elferink, 2002; Volk et al., 2002).

Antifolates use these physiological folate transport pathways to enter normal and malignant cells. RFC, in particular, is an important determinant of the activity of antifolates, best characterized for methotrexate (MTX). Impaired MTX transport caused by decreased RFC expression or altered RFC function (i.e., mutations) has been well documented in acquired resistance in a variety of antifolate-resistant murine and human tumor cell lines and tissues (Matherly and Goldman, 2003). Overexpression of MRP1–4 increases MTX transport into inverted membrane vesicles and suppresses MTX uptake into intact cells, resulting in acquired MTX

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ABBREVIATIONS: RFC, reduced folate carrier; BCRP, breast cancer resistance protein; MRP, multidrug resistance protein; MTX, methotrexate; CHO, Chinese hamster ovary; MK571, 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoylethylsulfanyl)methylsulfanyl] propionic acid; HEK, human embryonic kidney; DHFR, dihydrofolate reductase; AG2034, 4-[2-(2-amino-4-oxo-4,6,7,8-tetrahydro-3H-pyrimidino[5,4,6][1,4]thiazin-6-yl)-(*S*)-ethyl]-2,5-thienoylamino-L-glutamic acid; AG337, nolatrexed dihydrochloride.

resistance and increased folate growth requirement (Hooijberg et al., 1999; Kool et al., 1999; Lee et al., 2000; Zeng et al., 2001). Thus far, however, there is no example of selection with an antifolate resulting in the induction of primary resistance that is caused by MRP overexpression. In one case, however, mammalian cells selected for resistance to mitoxantrone because of overexpression of BCRP were cross-resistant to MTX, and this was associated with decreased drug accumulation (Volk et al., 2000, 2002).

It is clear that the level of endogenous physiological folates in cells is an important determinant of antifolate activity. Folate levels in murine leukemia cells are very sensitive to the extracellular level; because the extracellular folate concentration is increased from below to above the physiological range, there is a near-proportional increase in intracellular folate pools (Zhao et al., 2001). High intracellular folate levels inhibit the formation of active polyglutamate derivatives of antifolates that are retained in cells (Andreassi and Moran, 2002) and can compete with antifolates at the level of their target enzymes (Jackson and Harrap, 1973; White, 1979; White and Goldman, 1981). The contraction of folate pools within cells because of mutations in RFC, with concurrent impaired transport of reduced folates, can compensate for the loss of antifolate transport activity by enhancing the rate and extent of antifolate polyglutamation (Zhao et al., 2000a,b). On the other hand, RFC mutations that enhance transport of physiological folates and expand cellular folate levels result in resistance to antifolates (Tse et al., 1998; Tse and Moran, 1998).

The impact of cellular folate pools on the activity of antifolates is the basis for a novel mechanism of resistance observed in a Chinese hamster ovary (CHO) Pyr^{R100} cell line selected for resistance to the lipid-soluble antifolate pyrimethamine (Assaraf and Slotky, 1993). There was marked impairment of folate efflux with enhanced accumulation of physiological folates resulting in decreased activity of pyrimethamine and a variety of other antifolates (Assaraf and Goldman, 1997; Jansen et al., 1999). The current study was undertaken to characterize the basis for the loss of folate exporter function in Pyr^{R100} cells and to establish how this affected transport of a structurally different organic anion, cholate, an MRP substrate (Henderson et al., 1995; Jedlitschky et al., 1996).

Materials and Methods

Chemicals. [2,4-^{3}H]cholic acid (24.5 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). Folic acid, cholic acid (sodium salt), pyrimethamine, etoposide, vincristine, doxorubicin, and probenecid were obtained from Sigma Chemical Co. (St. Louis, MO). MK571 was obtained from Merck Frosst Canada (Kirkland, PQ, Canada), prostaglandin A_1 from Cayman Chemicals (Ann Arbor, MI), calcein AM from Molecular Probes (Eugene, OR), and G-418 from Calbiochem-Novabiochem (San Diego, CA).

Tissue Culture. A clonal subline (C11) of CHO AA8 cells was subjected to stepwise selection with increasing concentrations of pyrimethamine, resulting in the establishment of Pyr^{R100} cells as described previously (Assaraf and Slotky, 1993). Parental CHO AA8 cells and their Pyr^{R100} subline were maintained as monolayer cultures at 37°C in RPMI-1640 medium containing 5% fetal calf serum (Invitrogen, Carlsbad, CA), 2.3 μM folic acid, supplemented with 2 mM glutamine, 100 μg/ml penicillin/streptomycin, and 1 mM pyruvate. Human ovarian carcinoma 2008 cells and various sublines stably transduced with the human cDNA encoding for MRP1, MRP2, and MRP3, as well as HEK293 cells transfected with MRP5, were kindly provided by Prof. P. Borst and Dr. M. Kool (Netherlands Cancer Institute, Amsterdam, The Netherlands). HEK293/MRP4 and human breast carcinoma MCF-7/MR cells served as MRP4 and BCRP overexpressing controls, respectively. These human carcinoma cell lines were maintained in RPMI-1640 medium containing 2.3 μM folic acid, 10% fetal calf serum, 2 mM glutamine, and antibiotics.

Growth Inhibition Assay. Cells in mid-logarithmic growth were seeded in 24-well plates (8×10^3 and 10^4 cells/well, respectively) in medium (0.5 ml/well) containing various concentrations of pyrimethamine, MTX, doxorubicin, vincristine, cholic acid, etoposide, 6-mercaptopurine, or thioguanine. After 3 days, cells were detached by trypsinization, and viability was assessed by trypan blue exclusion. The IC_{50} is defined as the drug concentration at which growth was inhibited by 50% relative to untreated control cells.

[^{3}H]Cholic Acid Uptake. Exponentially growing cells were harvested by centrifugation (750*g* for 5 min), washed twice with HEPES-buffered saline transport buffer containing: 20 mM HEPES at pH 7.4, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 5 mM D-glucose supplemented with 1 mM pyruvate. Density was adjusted to 10^7 cells/ml in the same buffer. Transport measurements were performed as described previously (Assaraf and Goldman, 1997). Briefly, after 20-min incubation of cells at 37°C, uptake of [^{3}H]cholic acid (7600–8200 dpm/pmol) at a final concentration of 50 nM was initiated, after which 1-ml samples were drawn at the indicated times and transferred to centrifuge tubes containing 10 ml of ice-cold HEPES-buffered saline. Cells were then centrifuged and washed two additional times, after which the cell pellet was lysed, scintillation fluid was added, and radioactivity was measured.

[^{3}H]Cholic Acid Efflux. Wild-type AA8 and Pyr^{R100} cells were loaded with [^{3}H]cholic acid for 20 min at 37°C to comparable intracellular levels by incubation with buffer containing extracellular cholic acid concentrations of 150 and 50 nM, respectively. At the indicated times, cells were processed for the determination of intracellular radioactivity as described above.

Western Blot Analysis of MRP Expression. Microsomal proteins were extracted from 2×10^7 cells in a buffer (150 μl) containing: 50 mM Tris, pH 7.5, 50 mM β-mercaptoethanol, 0.5% Triton X-100, and the protease inhibitors aprotinin (60 μg/ml), leupeptin (5 μg/ml), phenylmethylsulfonyl fluoride (10 μg/ml), EDTA (1 mM), and EGTA (1 mM). After a 1-h incubation on ice, the extract was centrifuged at 15,000*g* for 30 min at 4°C, and the supernatant containing the fraction of detergent-soluble proteins was collected. Proteins (25–100 μg) were resolved by electrophoresis on 7% polyacrylamide gels containing SDS and electroblotted onto a Protran membrane (Schleicher and Schuell). Blots were blocked for 1 h at room temperature in TBS buffer (10 mM Tris at pH 8.0, 150 mM NaCl) containing 20% skim milk and then reacted for 1 h at room temperature with anti-human MRP and BCRP monoclonal antibodies (kindly provided by Prof. R.J. Scheper, VU Medical Center, Amsterdam, The Netherlands). These included various monoclonal antibodies (Maliepaard et al., 2001): rat anti-human MRP1 (MRPr1 at a 1:1000 dilution, 1-h incubation at room temperature), MRP5 (M_5I-1, 1:750 dilution, 1-h incubation) (Maliepaard et al., 2001), as well as mouse anti-human MRP1 (MRPm5, 1:500 dilution, 1-h incubation), MRP2 (M_2III-5, 1:50 dilution, overnight incubation at 4°C), MRP3 (M_3II-9, 1:500 dilution, 1-h incubation), MRP4 (M_4II-8, 1:15 dilution, overnight incubation), 2001) , and BCRP (BXP-21, 1:150 dilution, overnight incubation) in a TBS buffer containing 2% low fat milk and 0.1% Tween 20. Blots were then washed three times in TBS containing 0.5% Tween 20 for 10 min at room temperature, and then reacted with horseradish peroxidase-conjugated goat anti-mouse or anti-rat IgG (1:20,000 dilution; Jackson Immunoresearch Labs, West Grove, PA) for 1 h at room temperature. To examine whether the anti-human MRP and BCRP monoclonal antibodies cross-reacted with the hamster transporter proteins, liver and kidney microsomal proteins were extracted from fresh Syrian golden hamster (*Mesocricetus auratus*) tissues as

described previously (Assaraf and Borgnia, 1994). Na^+-K^+-ATPase (α subunit) served as an internal control and was detected overnight at 4°C with an affinity-purified rabbit polyclonal antiserum (anti-KETYY) at a 1:3000 dilution (kindly provided by Prof. S. J. D. Karlish, The Weizmann Institute of Science, Rehovot, Israel), followed by incubation with a second goat anti-rabbit IgG (1:6000). After three washes (10 min each) in TBS at room temperature, enhanced chemiluminescence detection was performed according to the manufacturer's instructions (Biological Industries, Beth Haemek, Israel). Protein content was determined using the Bio-Rad protein assay.

Flow Cytometric Analysis of Calcein AM Staining. Exponentially growing cells (10^6 cells/60-mm Petri dish) were incubated in growth medium (5 ml) containing 20 mM HEPES, pH 7.4, and 3 to 300 nM calcein AM, a chromophore that, in its intracellular anionic form (i.e., calcein), is an MRP1 (Olson et al., 2001) but not an MRP5 substrate (McAleer et al., 1999). After a 20-min incubation at 37°C, cells were harvested by centrifugation, washed once with phosphate-buffered saline, and analyzed for fluorescence intensity on a FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA). Excitation and emission were at 488 and 525 nm, respectively. Autofluorescence intensities of unstained AA8, Pyr^{R100}, and Pyr^{R100}/MRP5 cells were recorded and subtracted from those of calcein AM-stained cells.

Stable Transfections with hMRP1 and hMRP5 Expression Constructs. Exponentially growing Pyr^{R100} cells (2×10^7) in a pyrimethamine-free medium were harvested by centrifugation and stably transfected by electroporation (1000 μF, 234 V) and X-tremeGene reagent (Roche Applied Sciences, Indianapolis, IN) with 10 and 2 μg, respectively, of expression vectors containing the hMRP1 (pJ3Ω-MRP) and MRP5 (pGEM-MRP5) cDNA (kindly provided by Prof. P. Borst and Dr. M. Kool). After 24 h of growth at 37°C, cells were exposed to 400 to 600 μg/ml active G-418. Stable transfectants obtained after 2 months of G-418 selection were used for further analyses.

Results

Cross-Resistance Patterns in Pyrimethamine-Resistant Pyr^{R100} Cells. Previous studies demonstrated that CHO cells selected for resistance to pyrimethamine are cross-resistant to a variety of other antifolates, as indicated and referenced in Tables 1 and 2. This encompasses lipid-soluble DHFR inhibitors that enter cells by passive diffusion and do not form polyglutamate derivatives (metoprine, trimetrexate, piritrexim). Pyr^{R100} cells are also cross-resistant to the glycineamide ribonucleotide transformylase inhibitors 5,10-diadeazatetrahydrofolate and AG2034, good substrates for RFC but particularly sensitive to the level of endogenous folates in cells that inhibit their polyglutamation at the level of folylpolyglutamate synthetase (Zhao et al., 2000b, 2001). Cross-resistance to the lipid-soluble thymidylate synthase inhibitor AG337 was also noted. Interestingly, these cells displayed 3-fold resistance to thioguanine and 6-mercaptopurine, which inhibit purine synthesis by a mechanism distinct from, and independent of, folate pathways (Table 2).

Expression of MRPs in Wild-Type AA8 and Pyr^{R100} Cells. Previous studies demonstrated a marked loss of folate export activity in Pyr^{R100} cells, resulting in a substantial expansion in the intracellular folate pool (Assaraf and Goldman, 1997; Jansen et al., 1999). To explore the basis for the loss of folate efflux function, MRP1 through MRP5 and BCRP expression was examined in the microsomal fraction of wild-type AA8 and Pyr^{R100} cells (Fig. 1). Whereas wild-type cells expressed substantial levels of MRP1, Pyr^{R100} cells had no MRP1 expression on Western blot analysis performed with two different monoclonal antibodies (Fig. 1A). Furthermore, whereas wild-type AA8 cells expressed MRP5, albeit to a lesser extent than MRP1, Pyr^{R100} cells had barely detectable levels of MRP5 (10% of the level of AA8 cells; Fig. 1A). BCRP was clearly recognized by the specific monoclonal antibody used but was not detectable in either wild-type AA8 or in Pyr^{R100} cells (Fig. 1B). Expression of MRP2 was negligible in both wild-type and resistant cells with a very strong hamster liver positive control. The antibodies to MRP3 and MRP4 reacted only weakly to the hamster liver and kidney controls, respectively, and were not different from the intensity of the wild-type and Pyr^{R100} bands.

[^{3}H]Cholic Acid Efflux and Net Accumulation in AA8 and Pyr^{R100} Cells; Effects of Prostaglandin A_1. MRP1–4 transport a variety of organic anions, and MRP1–3 are known to transport bile salts, including cholic acid as well as their conjugates (Henderson et al., 1995; Jedlitschky et al., 1996). As indicated in Fig. 2, net cholic acid efflux from Pyr^{R100} cells loaded with [^{3}H]cholic acid was markedly reduced compared with efflux from wild-type AA8 cells ($T_{1/2}$ of 7.0 ± 0.2 versus 1.7 ± 0.1 min, respectively). As indicated in the inset, efflux of the major portion of intracellular [^{3}H]cholic acid can be characterized by a single exponential with a rate constant in Pyr^{R100} cells that was 25% of that of wild-type cells (k = 0.075 ± 0.002 versus 0.306 ± 0.008/min, respectively). This reduction in cholic acid efflux was associated with a ~6-fold higher net accumulation of cholate in Pyr^{R100} cells relative to wild-type AA8 cells (0.794 ± 0.081 versus 0.136 ± 0.02 nmol/g of dry weight, respectively (Fig.

TABLE 1
Cytotoxicity of various drugs to Pyr^{R100} and their parental AA8 cells
The IC_{50} values were obtained using a 72-h growth inhibition or colony formation assays. Results are the mean ± S.D. of three to six experiments.

Drug	Cell Line (IC_{50})		Resistance	Reference
	AA8	Pyr^{R100}		
	μM		*-fold*	
Metoprine	0.012 ± 0.004[a]	27.5 ± 4.5	2292	Assaraf and Slotky (1993)
Trimetrexate	10 ± 4	401 ± 39	40.1	Jansen et al. (1999)
AG337	27 ± 14	820 ± 100	30.4	Jansen et al. (1999)
5,10-Diadeazatetrahydrofolate	62 ± 36	1600 ± 1065	25.8	Jansen et al. (1999)
Piritrexim	0.0043 ± 0.0012	0.1 ± 0.02	23.3	Assaraf and Slotky (1993)
AG2034	28 ± 11	402 ± 222	14.4	Jansen et al. (1999)
Etoposide	0.21 ± 0.009	0.12 ± 0.004	0.6	Current study
Doxorubicin	0.04 ± 0.008	0.012 ± 0.005	0.3	Current study
Vincristine	0.008 ± 0.003	0.004 ± 0.002	0.5	Current study
Cholic acid	543 ± 55	187 ± 66	0.3	Current study

3A). Furthermore, net uptake of cholic acid was increased in wild-type AA8 cells to levels comparable with those obtained with Pyr^{R100} cells in the presence of 50 μM prostaglandin A_1 (PGA_1) (Fig. 3B), an MRP substrate (Henderson et al., 1995; Evers et al., 1997), and a potent inhibitor of folate and MTX export in CHO cells (Assaraf et al., 1999).

Cytotoxicity of Cholic Acid and Various MRP1 Substrates to AA8 and Pyr^{R100} Cells. Cholic acid at high concentrations is cytotoxic to mammalian cells. Consistent with the low rates of cholic acid efflux and high levels of net cholic acid accumulation, the cholate IC_{50} for inhibition of the growth of Pyr^{R100} cells was ~34% of the concentration for wild-type cells (187 ± 66 versus 543 ± 55 μM, respectively), as indicated in Table 1. Furthermore, consistent with the loss of MRP1 expression, Pyr^{R100} cells were up to 3-fold more sensitive to other cytotoxic MRP1 substrates—etoposide, doxorubicin, and vincristine—than wild-type AA8 cells (Borst and Oude Elferink, 2002).

TABLE 2

Growth inhibitory effect of various drugs in AA8, Pyr^{R100} and Pyr^{R100}/MRP5 cells

IC_{50} values were determined after 72 h of drug exposure. Results are the mean ± S.D. of three to five experiments.

Drug	Cell Line (IC_{50})		
	AA8	Pyr^{R100}	Pyr^{R100}/MRP5
		nM	
Pyrimethamine	0.13 ± 0.01[a]	122 ± 9	129 ± 14
MTX	11.5 ± 4.2	15.8 ± 5.8	15.7 ± 8.5
Thioguanine	635 ± 67	2184 ± 189	557 ± 101
6-Mercaptopurine	476 ± 135	1508 ± 279	223 ± 57

Flow Cytometric Analysis of Calcein AM Uptake in AA8 and Pyr^{R100} Cells. The anionic chromophore calcein is an MRP1 (Olson et al., 2001) but not an MRP5 substrate (McAleer et al., 1999) and is therefore a useful probe for deciphering the impact of the loss of MRP1 in cells in which expression of both exporters is reduced. Both cell lines showed a concentration-dependent increase in intracellular calcein fluorescence, but this was much greater for the Pyr^{R100} cells. The fluorescence ratio of Pyr^{R100} to AA8 reached a peak of ~5 at an extracellular calcein AM concentration of 100 nM (Fig. 4A). Furthermore, 2 mM probenecid, an MRP inhibitor, markedly increased calcein fluorescence in wild-type AA8 cells to levels comparable with those obtained with Pyr^{R100} cells but failed to alter the high level of calcein fluorescence in Pyr^{R100} cells (Fig. 4B). Finally, calcein fluorescence in Pyr^{R100} cells transfected with MRP5 (Pyr^{R100}/MRP5) was identical to that of nontransfected Pyr^{R100} cells (Fig. 4C).

The Impact of MRP1 and MRP5 Transfection on the Cytotoxicity of Pyrimethamine and MTX in Pyr^{R100} Cells. The loss of MRP1 expression and the marked decrease in the MRP5 level in Pyr^{R100} cells prompted studies to assess the impact of overexpression of these exporters on the cytotoxicity of pyrimethamine and MTX to Pyr^{R100} cells. Pyr^{R100} cells were transfected with expression constructs harboring MRP1 or MRP5 cDNAs, and stable transfectants (Pyr^{R100}/MRP1 and Pyr^{R100}/MRP5, respectively) growing in G-418

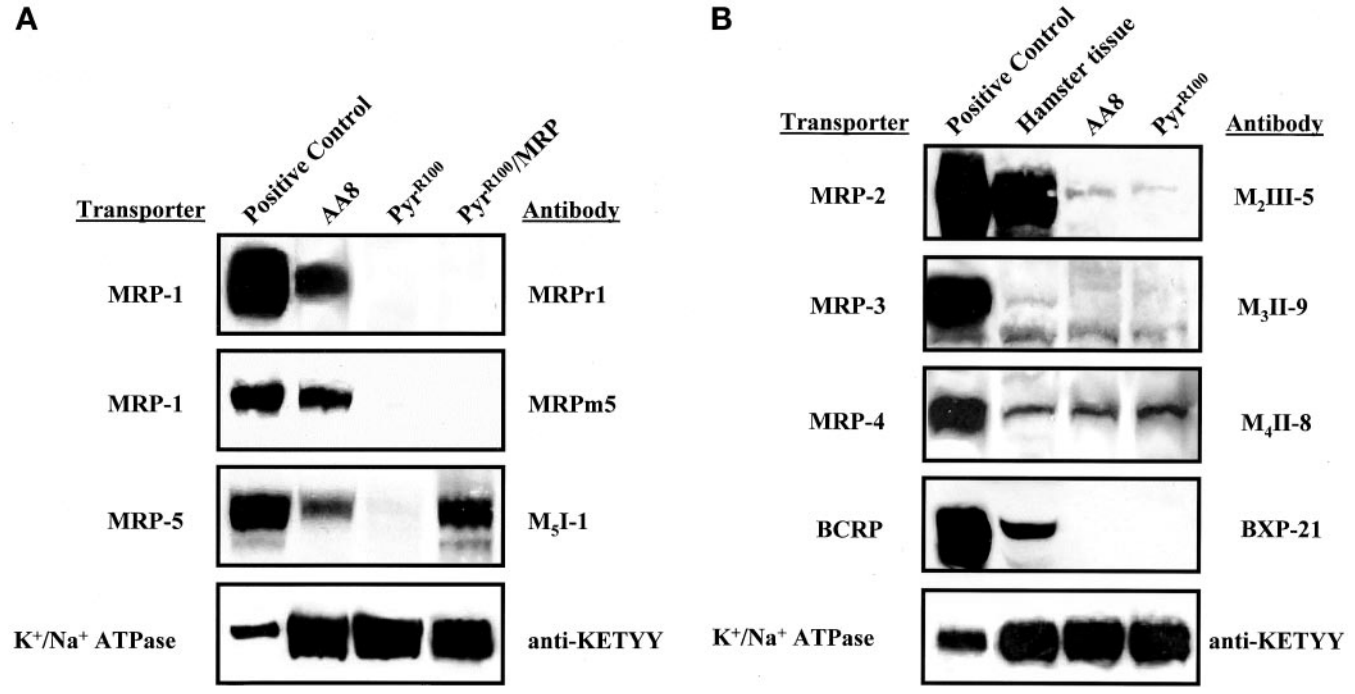

Fig. 1. Western blot analysis of MRP and BCRP expression in wild-type AA8 and Pyr^{R100} cells. Microsomal proteins from AA8, Pyr^{R100} (100 μg), human ovarian carcinoma 2008 cells transduced with MRP1 (A), MRP2, MRP3, MRP4 cDNAs (B), HEK293/MRP5 (A), and BCRP-overexpressing human breast carcinoma MCF-7/MR cells (B), were resolved by electrophoresis on 7% polyacrylamide gels, electroblotted onto Protran membranes, reacted with monoclonal antibodies to MRP1–5 and BCRP, and detected by enhanced chemiluminescence as detailed under *Materials and Methods*. Microsomal proteins (25 μg) from MRP1–5– and BCRP-overexpressing cells are shown in the "Positive control" lane (A and B), whereas freshly isolated microsomal proteins from Syrian golden hamster liver (100 μg for MRP2, -3, and BCRP) or kidney (100 μg for MRP4) are shown in the "Hamster tissue" lane (B). The Pyr^{R100}/MRP lanes (far right in A) represent microsomal proteins from Pyr^{R100} cells transfected with MRP1 or MRP5 cDNAs. The various monoclonal antibodies used are indicated on the right under "Antibodies," whereas the specific transporter studied is shown on the left under "Transporter" (A and B). Loading was assessed with an antibody to the α-subunit of Na^+-K^+-ATPase (bottom lanes, A and B). The blot shown is representative of five to seven independent experiments performed with different extracts.

were isolated. Pyr^{R100}/MRP5 cells expressed high levels of MRP5, relative to Pyr^{R100} and wild-type AA8 cells (Fig. 1A); however, the IC_{50} values for pyrimethamine with Pyr^{R100}/MRP5 and Pyr^{R100} cells were not different (129 ± 14 versus 122 ± 9 μM, respectively; Fig. 5 and Table 2). Sensitivity of Pyr^{R100}/MRP5 cells to MTX was also not different from that of Pyr^{R100} cells (Table 2). Activity of MRP5 in the transfected cells was confirmed by the observation that sensitivity to thioguanine and 6-mercaptopurine was restored. On the other hand, although cells transfected with the MRP1 construct displayed high-level resistance to 600 μg/ml G-418, in five separate transfections in which five to seven independent clones were studied, no expression of MRP1 could be detected (Fig. 1A).

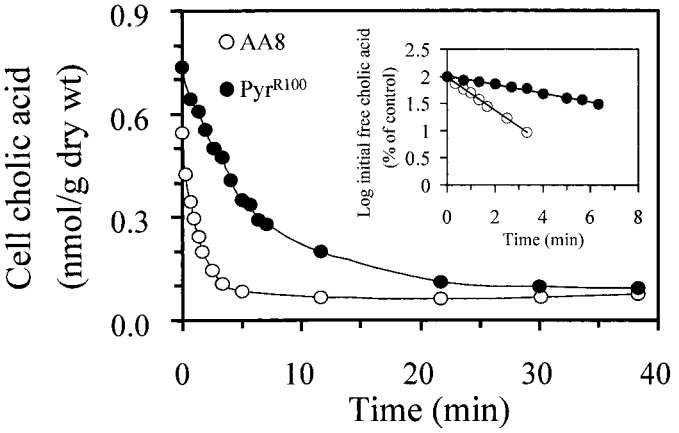

Fig. 2. Time course of cholic acid efflux in wild-type AA8 and Pyr^{R100} cells. After 20 min of loading to comparable intracellular [^{3}H]cholic acid levels, cells were harvested by centrifugation, resuspended into fresh buffer, and cell cholate was monitored as detailed under *Materials and Methods*. The inset indicates the log of the percentage of initial free cholic acid as function of time after resuspension into fresh buffer. The slope of the line is equal to the efflux rate constant (Assaraf and Goldman, 1997). Results are representative of three independent determinations.

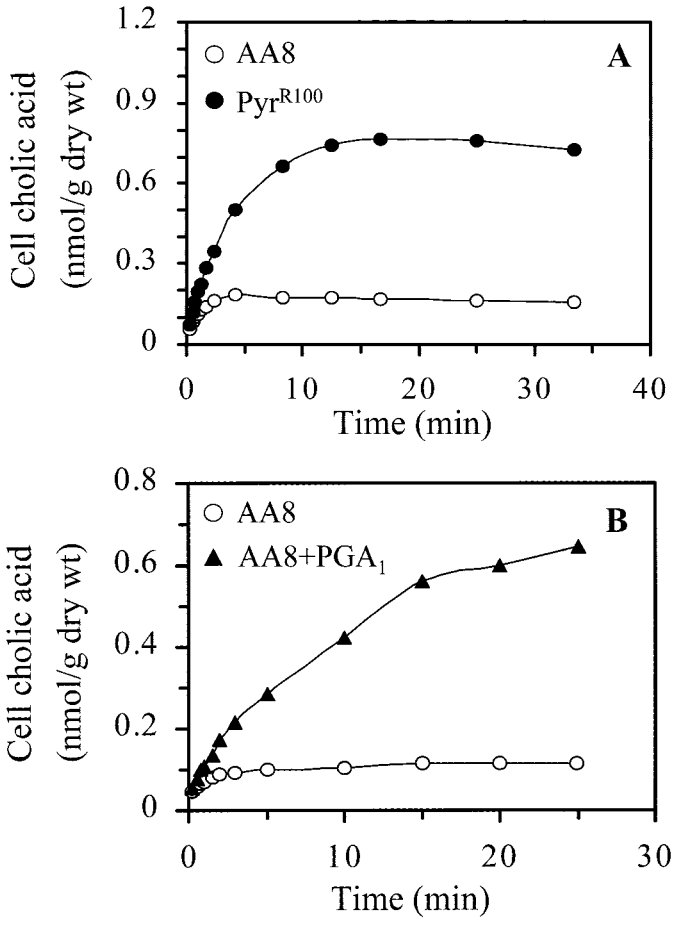

Fig. 3. A, time course of cholic acid uptake (50 nM) in wild-type AA8 and Pyr^{R100} cells. B, effect of PGA_1 on net [^{3}H]cholic acid uptake in wild-type AA8 cells. PGA_1 (50 μM) was added 3 min before the addition of [^{3}H]cholate. The net levels of cholic acid accumulation were determined from three to six experiments performed on different days.

Discussion

Acquired resistance to pyrimethamine in CHO Pyr^{R100} cells is associated with markedly impaired folate efflux resulting in a large increase in the net accumulation of physiological folates (Assaraf and Slotky, 1993; Assaraf and Goldman, 1997; Jansen et al., 1999). This, in turn, suppresses the interaction between pyrimethamine and its target enzyme dihydrofolate reductase, resulting in high-level resistance to this and other antifolate agents. A variety of observations in the current study indicate that this marked decrease in folate exporter activity in Pyr^{R100} cells is caused by the loss of MRP1 function:

1. MRP1 expression, as assessed with two monoclonal antibodies, was absent.
2. Export of cholic acid, which, along with its glycoconjugate, is an MRP substrate (Henderson et al., 1995; Jedlitschky et al., 1996) was markedly impaired in Pyr^{R100} cells and this resulted in a marked increase in net cholate accumulation.
3. PGA_1, an MRP1 substrate (Henderson et al., 1995; Evers et al., 1997) and a potent inhibitor of folate export (Assaraf et al., 1999) markedly increased net uptake of cholate in wild-type AA8 cells to levels comparable with that of Pyr^{R100} cells.
4. Flow cytometry analysis indicated a marked increase in the steady-state intracellular level of calcein, an MRP1 substrate, in Pyr^{R100} cells (Olson et al., 2001).
5. Probenecid, an MRP inhibitor, increased calcein accumulation in wild-type AA8 cells but had no effect on Pyr^{R100} cells.
6. Pyr^{R100} cells were collaterally sensitive to doxorubicin, etoposide, and vincristine—all transport substrates for MRP1 (Borst and Oude Elferink, 2002).

An attempt was made to restore the MRP1 phenotype to that of wild-type CHO cells. However, multiple transfections with the selection of multiple clones using an expression vector harboring MRP1 cDNA, by both electroporation and by the liposomal X-tremeGene reagent, resulted in high-level resistance to G-418 but no detectable expression of MRP1. Because the vector contained both the neomycin phosphotransferase resistance and the MRP1 genes, the failure of MRP1 expression must have been caused by either deletion of the MRP1 gene or some other mechanism by which its expression was silenced. Very high MRP1 expression might so deplete cell folates that survival of these clones would be impossible. In other studies, cellular folate levels were decreased in MRP1 transfectants, but this was not of sufficient

magnitude to alter the survival of these cells (Hooijberg et al., 2003).

In addition to the decrease in MRP1 expression in Pyr^{R100} cells, there was a marked decrease in expression of MRP5. This was unexpected because MRP5 does not seem to transport folates or antifolates. Rather, MRP5 transports 3′,5′-cyclic nucleotides, nucleotides, and nucleoside analogs and confers resistance to 6-mercaptopurine and thioguanine (Wijnholds et al., 2000). Furthermore, MTX does not inhibit cGMP transport in inverted erythrocyte membrane vesicles that are probably mediated, in part, by MRP5 (Sundkvist et al., 2002). Data in the current article are consistent with these observations and indicate that the loss of MRP5 does not contribute to the loss of folate export function in Pyr^{R100} cells. Hence, transfection and high-level expression of MRP5 in Pyr^{R100} cells had no effect on the level of resistance to pyrimethamine or MTX. However, sensitivity to thioguanine and 6-mercaptopurine was restored, confirming the activity of the transfected exporter.

Why MRP5 expression was lost during selection with pyrimethamine remains unclear. It is possible that this may have occurred because of the stringent conditions during the establishment of Pyr^{R100} cells; selection was performed with medium supplemented with only 5% *dialyzed* serum lacking ribonucleosides (Assaraf and Slotky, 1993). The continuous antifolate-mediated blockade of purine and thymidylate biosynthesis in the absence of exogenous nucleosides may have resulted in an adaptive down-regulation of this nucleotide transporter to preserve intracellular nucleotide pools. The expansion of these pools in Pyr^{R100} cells under usual growth conditions would probably result in impaired activation of nucleoside antimetabolites. Introduction of MRP5 into Pyr^{R100} cells restored wild-type sensitivity to thiopurines, presumably because of a decrease in ribonucleoside pools. Additional studies will be required to further substantiate this formulation.

Pyr^{R100} cells have undergone several adaptive changes that result in expansion of cellular folate pools: 1) the expression of at least one major ATP-driven folate efflux pathway, MRP1, was completely lost; 2) influx of folic acid was enhanced 4-fold because of increased activity of a low pH transporter (Assaraf et al., 1998), and 3) folylpolyglutamate synthetase activity, and hence the rate and extent of folate polyglutamylation, was increased 3- to 4-fold (Jansen et al., 1999). The latter results in the formation of congeners that are retained within cells and allow the build-up of high folate levels, because long-chain polyglutamate conjugates of folates are not substrates for MRP1-MRP4 (Zeng et al., 2001; Chen et al., 2002). Collectively, these alterations contribute to a marked increase in the net accumulation of folates,

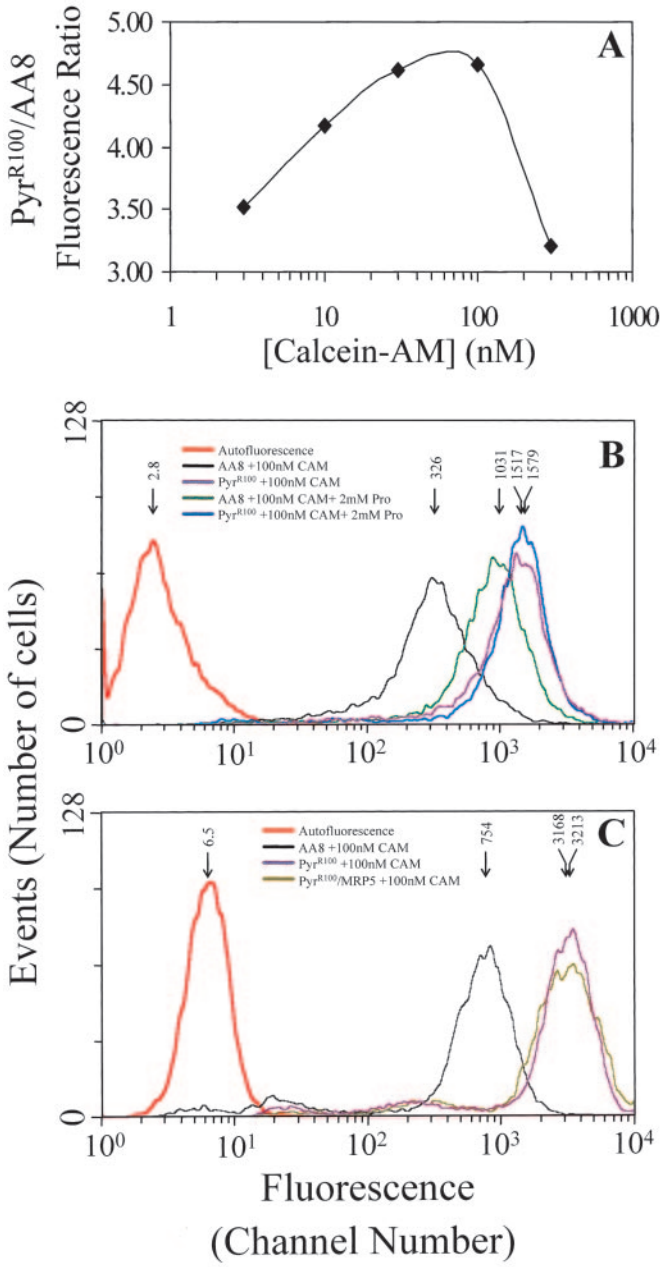

Fig. 4. Calcein-AM accumulation in AA8, Pyr^{R100}, and Pyr^{R100}/MRP5 cells. Exponentially growing cells were incubated for 20 min in growth medium in the absence or presence of 3 to 300 nM calcein AM, after which cells were washed and analyzed by flow cytometry. A, the fluorescence ratio of Pyr^{R100}/AA8 as a function of the extracellular calcein AM concentration. B, the effect of probenecid (2 mM) on the accumulation of calcein-AM (100 nM) in AA8 and Pyr^{R100} cells. *C*: The accumulation of calcein in the MRP5 transfectant, Pyr^{R100}/MRP5, as well as in Pyr^{R100} and AA8 cells. Data are representative of three independent experiments.

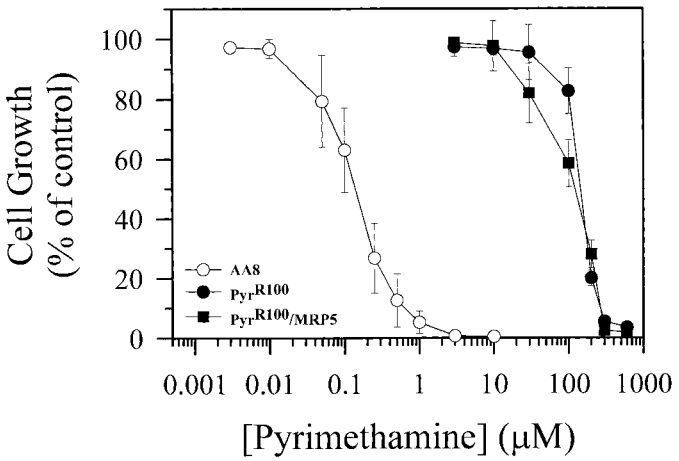

Fig. 5. Growth inhibitory effects of pyrimethamine on AA8, Pyr^{R100}, or Pyr^{R100}/MRP5 cells. Exponentially growing cells were exposed to various concentrations of pyrimethamine as detailed under *Materials and Methods*. After 3 days of incubation, viable cells were determined by trypan blue exclusion. The data are the mean ± S.D in three independent experiments.

resulting in a 100-fold decrease in the folic acid growth requirement in Pyr^{R100} cells relative to parental AA8 cells (Jansen et al., 1999).

Like the adaptive change in MRP1 expression that occurred in response to pyrimethamine-selective pressure, other studies indicate that a similar change can also occur in response to folate deprivation. When CEM-7A cells were subjected to gradual leucovorin deprivation, there was overexpression of RFC as well as loss of MRP1 expression (Jansen et al., 1990; Assaraf et al., 2003). Because increased RFC expression can produce near-symmetrical changes in influx and efflux of folates, with only small increases in steady-state free levels (Zhao et al., 1997), and because endogenous MRP activity always suppresses the free folate and antifolate monoglutamyl levels in cells (Assaraf et al., 1999; Zhao et al., 2000a), it is likely that the loss of MRP1 export is the most efficient way for cells to substantially enhance concentrative folate uptake.

Recent studies have established that augmentation of cellular tetrahydrofolate cofactor pools results in marked suppression of the activity of antifolates. Intracellular folate pools increase almost in proportion to the increase in extracellular folate concentration, even over the range of physiological blood folate levels. As the intracellular folate level is increased, because of an increase in extracellular folate (Zhao et al., 2001) or mutations in RFC that enhance folate transport (Tse et al., 1998; Tse and Moran, 1998), there are substantial decreases in the activities of antifolates that do and do not form polyglutamate derivatives. There are several mechanisms that underlie the decreased cytotoxic activity of antifolates that encompass resistance to antifolates observed in this and earlier studies with the Pyr^{R100} cell line:

1. In the case of the 4-amino antifolate inhibitors of DHFR, as this enzyme is suppressed, tetrahydrolate cofactors interconvert to dihydrofolate so that as tetrahydrofolate cofactor pools are increased, the levels of dihydrofolate achieved are increased. This results in increased competition between dihydrofolate and 4-amino antifolates at the level of DHFR (Jackson and Harrap, 1973; White, 1979; Seither et al., 1989). This is the mechanism of resistance to pyrimethamine and the other lipid-soluble DHFR inhibitors in Pyr^{R100} cells (Table 1) and to MTX (Table 2) (Zhao et al., 2001).
2. As the tetrahydrofolate cofactor pool is increased, there is feedback inhibition of polyglutamylation of antifolates with suppression of accumulation of these active antifolate derivatives (Andreassi and Moran, 2002). This is a very important factor in the observed resistance to 5,10-diadeazatetrahydrofolate and AG2034 (Tse et al., 1998; Tse and Moran, 1998; Zhao et al., 2000b, 2001).
3. As cellular tetrahydrofolate cofactor pools are increased, there may be increased competition between the physiological folate substrate and antifolate at the level of thymidylate synthase and/or glycinamide ribonucleotide transformylase. This would account for the high degree of resistance to AG337 (Table 1), a lipid-soluble inhibitor of thymidylate synthase that does not form polyglutamate derivatives (Webber et al., 1996).

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Address correspondence to: Dr. Yehuda G. Assaraf, Department of Biology, The Technion-Israel Institute of Technology, Haifa 32000, Israel. E-mail: assaraf@tx.technion.ac.il