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

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ABSTRACT

Export of folates from a Chinese hamster ovary PyrR100 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 PyrR100 cells along with a marked decrease in MRP5 expression with 3-fold cross-resistance to thiopurines. PyrR100 and wild-type cells had comparable low levels of MRP2 expression; both lacked the breast cancer resistance protein. PyrR100 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 A1 increased cholate accumulation in wild-type cells to levels comparable with PyrR100 cells. Calcein (an MRP1 substrate) fluorescence increased 5-fold in PyrR100 cells; probenecid increased the intracellular calcein level in wild-type cells to that of PyrR100 cells. Consistent with the loss of MRP1 expression, PyrR100 cells showed modest collateral sensitivity to cholate, etoposide, doxorubicin, and vincristine. Transfection of MRP5 into PyrR100 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). 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 endocytic 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

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-dimethylcarbamoylethylsulfonyl][methylsulfanyl] propionic acid; HEK, human embryonic kidney; DHFR, dihydrofolate reductase; AG2034, 4-[2-(2-amino-4-o xo-4,6,7,8-tetrahydro-3H-pyrimido[5,4,6][1,4]thiazin-6-yl) -(S)-ethyl] -2,5-thienoylamino-L-glutamic acid; AG337, nolatrexed dihydrochloride.

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resistance and increased folate growth requirement (Hooi-
berg 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 re-
sistance that is caused by MRP overexpression. In one case,
however, mammalian cells selected for resistance to mitox-
antrone because of overexpression of BCRP were cross-resis-
tant 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 con-
centration 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 lev-
els inhibit the formation of active polyglutamate derivatives of
antifolates that are retained in cells (Andreassi and Mo-
ran, 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 concur-
rent 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

The impact of cellular folate pools on the activity of anti-
folates is the basis for a novel mechanism of resistance ob-
served in a Chinese hamster ovary (CHO) PyrR100 cell line
selected for resistance to the lipid-soluble antifolate py-
rimethamine (Assaraf and Slotky, 1993). There was marked
impairment of folate efflux with enhanced accumulation of
physiological folates resulting in decreased activity of py-
rimethamine 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 PyrR100 cells and to establish how this
affected transport of a structurally different organic anion,
cholate, an MRP substrate (Henderson et al., 1995; Jedli-
schky et al., 1996).

Materials and Methods

Chemicals. [2,4-3H]cholic acid (24.5 Ci/nmol) 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,
Canada). Sodium pyruvate, cholate, and probenecid were obtained from Sigma Chemical Co. (St. Louis,
MO). MK571 was obtained from Merck Frosst Canada (Kirkland,
Canada). Sodium pyruvate, cholate, and probenecid were obtained from Sigma Chemical Co. (St. Louis,
MO).

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 PyrR100 cells
as described previously (Assaraf and Slotky, 1993). Parental CHO AA8
cells and their PyrR100 subline were maintained as monolayer cul-
tures in RPMI-1640 medium containing 5% fetal calf serum
(Invitrogen, Carlsbad, CA). 2.3 μM folic acid, supplemented with 2 mM
-glutamate, 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/MPR4 and hu-
man 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 × 103 and 104 cells/well, respectively) in
medium (0.5 ml/well) containing various concentrations of py-
rimethamine, 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 exclu-
sion. The IC50 is defined as the drug concentration at which growth
was inhibited by 50% relative to untreated control cells.

[3H]Cholic Acid Uptake. Exponentially growing cells were har-
vested by centrifugation (750g 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 MgCl2, and 5 mM
-glucose supplemented with 1 mM pyruvate. Density was adjusted
to 107 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 [3H]cholic
acid (7600–8200 dpm/ml) at a final concentration of 50 nM was
initiated, after which 1-m1 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.

[3H]Cholic Acid Efflux. Wild-type AA8 and PyrR100 cells were
loaded with [3H]cholic acid for 20 min at 37°C to comparable intra-
cellular 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 intra-
cellular radioactivity as described above.

Western Blot Analysis of MRP Expression. Microsomal pro-
teins were extracted from 2 × 107 cells in a buffer (150 μl) contain-
ing: 50 mM Tris, pH 7.5, 50 mM β-mercaptoethanol, 0.5% Triton
X-100, and the protease inhibitors aprotonin (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 centri-
fuged at 15,000g for 30 min at 4°C, and the supernatant contain-
ing the proteins was centrifuged at 25,000g for an additional 20 min at
4°C, and then dialyzed against 0.5 M ammonium sulfate. The
proteins (25–100 μg) were resolved by electrophoresis on 7% polyacryla
gels containing SDS and electrobotted onto a Protran membrane
(Schleicher and Schuell). Blots were blocked for 1 h at room tem-
perature 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 pro-
voked by Prof. R.J. Scheper, VU Medical Center, Amsterdam, TheNeth-
ernlands). These included various monoclonal antibodies (Maliepaad
et al., 2001), rat anti-human MRP1 (MRP1 at a 1:1000 dilution, 1-h
incubation at room temperature), MRP5 (M1-I-1, 1:750 dilution, 1-h
incubation) (Maliepaad et al., 2001), as well as mouse anti-human
MRP1 (MRP5-1, 1:500 dilution, 1-h incubation), MRP2 (M2-II-5, 1:500
dilution, overnight incubation at 4°C), MRP3 (M3-I-9, 1:500 dilution,
1-h incubation), MRP4 (M4-II-3, 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 di-
lution; 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 trans-
porter 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-KRTTY, at 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 (10⁶ 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 set at 488 and 525 nm, respectively. Autofluorescence intensities of unstained AA8, PyrR100, and PyrR100/MRP5 cells were recorded and subtracted from those of calcein AM-stained cells.

Stable Transfections with hMRP1 and hMRP5 Expression Constructs. Exponentially growing PyrR100 cells (2 × 10⁶) 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 (pJ3/H9024-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 PyrR100 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). PyrR100 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 PyrR100 Cells. Previous studies demonstrated a marked loss of folate export activity in PyrR100 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 microsomai fraction of wild-type AA8 and PyrR100 cells (Fig. 1). Whereas wild-type cells expressed substantial levels of MRP1, PyrR100 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, PyrR100 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 PyrR100 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 PyrR100 bands.

[H]Cholic Acid Efflux and Net Accumulation in AA8 and PyrR100 Cells; Effects of Prostaglandin A₁. 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; Jedditschky et al., 1996). As indicated in Fig. 2, net cholic acid efflux from PyrR100 cells loaded with [3H]cholic acid was markedly reduced compared with efflux from wild-type AA8 cells (T1/2 of 7.0 ± 0.2 versus 1.7 ± 0.1 min, respectively). As indicated in the inset, efflux of the major portion of intracellular [3H]cholic acid can be characterized by a single exponential with a rate constant in PyrR100 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 PyrR100 cells relative to wild-type AA8 cells (0.794 ± 0.081 versus 0.136 ± 0.02 nmol/g of dry weight, respectively (Fig.

<p>| TABLE 1 | Cytotoxicity of various drugs to PyrR100 and their parental AA8 cells | The IC₅₀ values were obtained using a 72-h growth inhibition or colony formation assays. Results are the mean ± S.D. of three to six experiments. |</p>
<table>
<thead>
<tr>
<th>Drug</th>
<th>Cell Line (IC₅₀)</th>
<th>AA8</th>
<th>PyrR100</th>
<th>Resistance</th>
<th>Reference</th>
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<tr>
<td></td>
<td>µM</td>
<td>26.75 ± 4.5</td>
<td>2292</td>
<td>Jansen et al. (1999)</td>
<td></td>
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<tr>
<td></td>
<td>µM</td>
<td>40.1</td>
<td>40.1</td>
<td>Jansen et al. (1999)</td>
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<tr>
<td></td>
<td>µM</td>
<td>30.4</td>
<td>30.4</td>
<td>Jansen et al. (1999)</td>
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<td></td>
<td>µM</td>
<td>25.8</td>
<td>25.8</td>
<td>Jansen et al. (1999)</td>
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<td></td>
<td>µM</td>
<td>22.3</td>
<td>22.3</td>
<td>Jansen et al. (1999)</td>
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<tr>
<td></td>
<td>µM</td>
<td>14.4</td>
<td>14.4</td>
<td>Jansen et al. (1999)</td>
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<td></td>
<td>µM</td>
<td>0.6</td>
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<td>Current study</td>
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<td>Current study</td>
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<td>Current study</td>
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</table>
Furthermore, net uptake of cholic acid was increased in wild-type AA8 cells to levels comparable with those obtained with PyrR100 cells in the presence of 50 μM prostaglandin A₁ (PGA₁) (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 PyrR100 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 IC₅₀ for inhibition of the growth of PyrR100 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, PyrR100 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).

**Flow Cytometric Analysis of Calcein AM Uptake in AA8 and PyrR100 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 PyrR100 cells. The fluorescence ratio of PyrR100 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 PyrR100 cells but failed to alter the high level of calcein fluorescence in PyrR100 cells (Fig. 4B). Finally, calcein fluorescence in PyrR100 cells transfected with MRP5 (PyrR100/ MRP5) was identical to that of nontransfected PyrR100 cells (Fig. 4C).

**The Impact of MRP1 and MRP5 Transfection on the Cytotoxicity of Pyrimethamine and MTX in PyrR100 Cells.** The loss of MRP1 expression and the marked decrease in the MRP5 level in PyrR100 cells prompted studies to assess the impact of overexpression of these exporters on the cytotoxicity of pyrimethamine and MTX to PyrR100 cells. PyrR100 cells were transfected with expression constructs harboring MRP1 or MRP5 cDNAs, and stable transfectants (PyrR100/ MRP1 and PyrR100/MRP5, respectively) growing in G-418

<table>
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<tr>
<th>Drug</th>
<th>Cell Line (IC₅₀)</th>
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<tr>
<td></td>
<td>AA8</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>0.13 ± 0.01*</td>
</tr>
<tr>
<td>MTX</td>
<td>11.5 ± 4.2</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>635 ± 67</td>
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<tr>
<td>6-Mercaptopurine</td>
<td>476 ± 135</td>
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**Table 2**

Growth inhibitory effect of various drugs in AA8, PyrR100 and PyrR100/ MRP5 cells

IC₅₀ values were determined after 72 h of drug exposure. Results are the mean ± S.D. of three to five experiments.

**Fig. 1.** Western blot analysis of MRP and BCRP expression in wild-type AA8 and PyrR100 cells. Microsomal proteins from AA8, PyrR100 (100 μg), human ovarian carcinoma 2008 cells transduced with MRP1 (A), MRP2, MRP3, MRP4 cDNAs (B), HEK293/MPR5 (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 PyrR100/MPR lanes (far right in A) represent microsomal proteins from PyrR100 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. PyrR100/MRP5 cells expressed high levels of MRP5, relative to PyrR100 and wild-type AA8 cells (Fig. 1A); however, the IC50 values for pyrimethamine with PyrR100/MRP5 and PyrR100 cells were not different (129 ± 14 versus 122 ± 9 μM, respectively; Fig. 5 and Table 2). Sensitivity of PyrR100/MRP5 cells to MTX was also not different from that of PyrR100 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).

**Discussion**

Acquired resistance to pyrimethamine in CHO PyrR100 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 PyrR100 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 PyrR100 cells and this resulted in a marked increase in net cholate accumulation.
3. PGA1, 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 PyrR100 cells.
4. Flow cytometry analysis indicated a marked increase in the steady-state intracellular level of calcein, an MRP1 substrate, in PyrR100 cells (Olson et al., 2001).
5. Probenecid, an MRP inhibitor, increased calcein accumulation in wild-type AA8 cells but had no effect on PyrR100 cells.
6. PyrR100 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 PyrR100 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 PyrR100 cells. Hence, transfection and high-level expression of MRP5 in PyrR100 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 PyrR100 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 PyrR100 cells under usual growth conditions would probably result in impaired activation of nucleoside antimetabolites. Introduction of MRP5 into PyrR100 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.

PyrR100 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, PyrR100, and PyrR100/MPR5 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 PyrR100/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 PyrR100 cells. C, The accumulation of calcein in the MRP5 transfectant, PyrR100/MPR5, as well as in PyrR100 and AA8 cells. Data are representative of three independent experiments.

**Fig. 5.** Growth inhibitory effects of pyrimethamine on AA8, PyrR100, or PyrR100/MPR5 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 PyrR100 cells relative to parental AAS 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 over-expression 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 PyrR100 cell line:

1. In the case of the 4-amino antifolate inhibitors of DHFR, as this enzyme is suppressed, tetrahydrofolate 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 PyrR100 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 glycaminide 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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