Antifolate Resistance Mediated by the Multidrug Resistance Proteins MRP1 and MRP2

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Abstract

Transfection of multidrug resistance proteins (MRPs) MRP1 and MRP2 in human ovarian carcinoma 2008 cells conferred a marked level of resistance to short-term (1–4 h) exposure to the polyglutamatable antifolates methotrexate (MTX; 21–74-fold), ZD1694 (4–138-fold), and GW1843 (101–156-fold). Evidence for MRP-mediated antifolate efflux relies upon the following findings: (a) a 2–3.3-fold lower accumulation of [3H]MTX and subsequent reduced formation of long-chain polyglutamate forms of MTX; (b) reversal of MTX resistance by probenecid in both transfected, and (c) ATP-dependent uptake of [3H]MTX in inside-out vesicles of MRP1 and MRP2 transfectedants. This report provides a mechanistic basis for resistance to polyglutamatable antifolates through an MRP-mediated drug extrusion.

Introduction

Tumor cell resistance to a wide spectrum of anticancer agents continues to be a major obstacle to curative cancer chemotherapy. This resistance, referred to as multidrug resistance, may result from the overexpression of MRP1, which can confer cellular resistance to natural product drugs, including anthracyclines, Vinca alkaloids, and epipodophyllotoxins (1–3). MRP1 belongs to the superfamily of ATP binding cassette transporter proteins (4) and mediates the ATP-driven unidirectional transport of a broad range of neutral as well as anionic substrates for MRP.

MRP4 is considered to be primarily a multispecific organic anion transporter (5). At least five homologues of MRP1 (i.e., MRP2–MRP6) have been identified (6, 7).

The folate analogue MTX3 is an anticancer agent that is used in treatment regimens for childhood leukemia, head and neck cancer, breast cancer, and osteogenic sarcoma (8). MTX is a potent inhibitor of dihydrofolate reductase, a key enzyme in the metabolism of reduced folate cofactors, which are also required for the biosynthesis of purines (8). Several mechanisms of resistance to MTX and other antifolates have been described, including (a) DHFR overexpression, (b) mutated and kinetically altered DHFR, (c) low expression and deficient inward transport via the RFC, and/or (d) decreased polyglutamylation of antifolates due to a lowered activity of FPGS (8–10). Novel antifolates, including the thymidylate synthase inhibitors ZD1694 (11) and GW1843U89 (12), have been recently introduced in an attempt to overcome some of these resistance modalities by more efficient RFC-mediated cellular uptake and/or polyglutamylation (13).

Although increased efflux of MTX has been considered to be a potential mechanism of resistance, thus far, no studies have implicated the increased expression of a specific organic anion efflux pump, e.g., MRP, in tumor cell resistance to antifolate drugs. Recently, however, Masuda et al. (14) reported that MRP2 plays a role in the excretion of MTX into the bile, suggesting that MTX and other antifolates may be substrates for MRP.

We show here that stable transfection of MRP1 and MRP2 cDNA confers a marked resistance to polyglutamatable antifolate drugs, including MTX, GW1843, and ZD1694. The resistance phenotype is predominantly observed after short-term (i.e., clinically relevant) drug exposure, which does not provide sufficient time to convert these drugs into long-chain polyglutamate forms.

Materials and Methods

Chemicals. MTX was a gift from Pharmachemie (Haarlem, the Netherlands), and [3H]MTX (15 Ci/mmol) was from Moravek Biochemicals (Brea, CA) and purified prior to use, as described previously (15). ZD1694 was obtained from Dr. F. T. Boyle (Zeneca Pharmaceuticals, Macclesfield, United Kingdom), GW1843U89 was a gift from Dr. G. K. Smith (Glaxo Wellcome, Research Triangle Park, NC), and trimetrexate was a gift from Warner-Lambert/Parke Davis (Ann Arbor, MI). Probenecid, ATP, and phenylmethylsulfonyl fluoride were from Sigma Chemical Co. (St. Louis, MO). Doxorubicin hydrochloride was from Laboratoire Roger Bellon (France).

Cell Lines. The human ovarian carcinoma cell line 2008 and its stable MRPs and MRP2 (i.e., canalicular multispecific organic anion transporter) transfectedants 2008/MP1 (clone 6) and 2008/MP2 (clone 7) were cultured in RPMI 1640 (Flow Labs, Irvine, United Kingdom), supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco, Paisley, United Kingdom), 2 mM glutamine, and 100 μg/ml penicillin and streptomycin.

The expression of MRPs and MRP2 in 2008 transfected cells (which will be described in detail elsewhere)3 was determined in immunocytochemical staining experiments using monoclonal MRP1 antibody against MRP1 (16), monoclonal M,-III-6 antibody against MRP2 (17), and Imr94 antibody (IgG2a) as a control (18). Compared to 2008 cells, the MRP1-transfected cells showed markedly increased expression of MRP1, which was predominantly localized in the plasma membrane. MRP2-transfected cells showed a high MRP2 expression, mainly in the cytosol but also, to a significant level, in the plasma membrane.

Plasma Membrane Vesicles. Inside-out plasma membrane vesicles were prepared from all cell lines as described previously (19), with slight modifications. Cells were harvested by centrifugation (275 × g, 5 min) and washed twice in ice-cold PBS (pH 7.4). The cell suspension (107 cells/ml) was incubated in a buffer containing 100 mM KCl, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 50 mM HEPES/KOH (pH 7.4) for 60 min at 0°C, followed by sonication at 20% of the maximum power of an M.S.E. sonicator (Soniprep 150) for three bursts of 15 s each. The suspension was centrifuged

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3 The abbreviations used are: MTX, methotrexate; DHFR, dihydrofolate reductase; RFC, reduced folate carrier; FPGS, folylpolyγ-glutamate synthetase; MRP, multidrug resistance protein; HBS, HEPES-buffered saline.

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at 1500 × g for 10 min. The postnuclear supernatant was layered on top of a 46% sucrose cushion and centrifuged at 100,000 × g for 60 min. The interface was removed and washed in the buffer described above. The final membrane preparations were stored at −80°C at a protein concentration of −4 mg/ml. The enrichment of Na+/K+ ATPase activity was −5-fold (19).

Uptake of [3H]MTX into Inside-out Plasma Membrane Vesicles. Transport of [3H]MTX into isolated inside-out plasma membrane vesicles was measured by rapid filtration as described previously (19, 20). Vesicles were incubated in a buffer containing 100 mM KCl-50 mM HEPES/KOH (pH 7.4) at 37°C (−0.25 mg/ml protein), in the presence of 10 mM MgCl2, 1 mM ATP, and 0.1 μM [3H]MTX (specific activity, 0.5 Ci/mmol) in a final reaction volume of 50 μl. The transport was stopped after 10 min by the addition of 2 ml of ice-cold KCl/HEPES buffer, followed by rapid filtration through O/E67 membrane filters (Schleicher & Schuell, Dassel, Germany). The filters were washed twice with 2 ml of KCl/HEPES buffer. The radioactivity associated with the filters was measured by liquid scintillation counting.

[3H]MTX Accumulation and Polyglutamylation. 2008, 2008/MRP1, and 2008/MRP2 cells were grown as monolayer cells in 80-cm² flasks until 60% confluent. Cells were resuspended in 1 ml of HBS, of which 10−6 M [3H]MTX (specific activity, 0.5 Ci/mmol) for a period of 4 and 24 h. Following these incubation times, cells were washed twice with 10 ml of ice-cold HBS (pH 7.4), trypsinized, and collected in 10 ml of ice-cold HBS. After centrifugation, cells were resuspended in 1 ml of HBS, of which 10−6 M aliquots were used for cell counting, 100 μl were used for radioactivity counting, and 890 μl were used for [3H]MTX-polyglutamate analysis by high-performance liquid chromatography as described by Westerhof et al. (15).

Growth Inhibition Assays. Cells were plated in 1 ml of medium at an initial density of 1 × 10⁶ cells/ml in individual wells of a 24-well plate (Nunc). One day after cell plating, drugs were added at 10 different concentrations covering a 3-log concentration range. Cells were exposed to drugs either continuously for 72 h or for a short-term (i.e., 1–4 h). After short-term exposure, the medium was aspirated, and cells were washed three times with 2.5 ml of drug-free medium at 37°C, whereas after 72 h, cells were collected by trypsinization and counted using a micro-cell counter, as described previously (15).

Results

ATP-dependent [3H]MTX Transport into Inside-out Plasma Membrane Vesicles. Plasma membrane inside-out vesicles of 2008/MRP1 and 2008/MRP2 cells were used to assess MRP1- and MRP2-mediated uptake of [3H]MTX (Fig. 1). To investigate MRP1-dependent MTX transport, we used the neutralizing MIB6 monoclonal antibody directed to an internal epitope of MRP (20). Fig. 1 shows that the ATP-dependent uptake of [3H]MTX into MRP1-rich inverted vesicles was inhibited completely by MIB6 (0.9 ± 0.2 versus 0.3 ± 0.2 pmol/mg of protein/min), thus approximating the ATP-independent component. Uptake of [3H]MTX into vesicles from the MRP2-expressing cell line was also ATP dependent (0.6 ± 0.1 pmol/mg of protein/min) but could not be blocked by the MIB6 antibody. The results obtained with the 2008/MRP1 vesicles were fully reproduced with vesicles isolated from GLC4/ADR cells, a human lung GLC4 cell line overexpressing MRP1 (Ref. 2; results not shown).

Growth Inhibition by Antifolates of Parental 2008 Cells and Their MRP1 and MRP2 Transfected. Because the results from Fig. 1 indicated that MTX is a substrate for MRP1 and MRP2, we investigated whether overexpression of MRP1 and MRP2 can confer resistance to MTX in 2008/MRP1 and 2008/MRP2 cells. In addition to MTX, we also tested two novel antifolates, the thymidylate synthase inhibitors ZD1694 and GW1843. The rationale for using these two antifolates was to evaluate the role of differential polyglutamylase efficiency in drug sensitivity. MTX is slowly converted to long-chain polyglutamates and not further than the diglutamate form (12), whereas ZD1694 is rapidly converted to long-chain polyglutamates (11).

We first determined the growth-inhibitory effects of the antifolates after 72 h of continuous drug exposure (Table 1). 2008/MRP1 cells displayed a low-level resistance to MTX (1.9-fold), ZD1694 (2.6-fold), and GW1843 (4.0-fold) compared to 2008 cells, whereas 2008/MRP2 cells were almost as equally antifolate sensitive as their parental 2008 cells. It is of interest to note that both 2008/MRP1 and 2008/MRP2 cells were collaterally sensitive to trimetrexate, a lipophilic DHFR inhibitor.

In contrast to the 72 h exposure, when antifolate drug sensitivity was determined after a short-term drug exposure (i.e., 4 h), 2008/MRP1 and 2008/MRP2 cells were highly resistant to MTX (>78-fold and 21-fold, respectively) and GW1843 (156-fold and 101-fold, respectively) but not to ZD1694. However, when incubations with ZD1694 were further shortened to 1 h, a marked level of resistance to ZD1694 was seen, especially with 2008/MRP1 cells (138-fold; Table 1).

Probenecid is a well-established inhibitor of MRP activity (21) and of MTX efflux (21, 23). The addition of a nontoxic concentration (0.5 mM) of probenecid during the 4 h exposure to MTX almost completely reversed the MTX resistance phenotype observed with 2008/MRP1 and 2008/MRP2 cells (Table 1). When probenecid was also present during the drug-free period of 72 h, IC50 for MTX for 2008/MRP1 and 2008/MRP2 cells were similar to those of parental 2008 cells (Table 1).

Cellular Accumulation and Polyglutamylation of [3H]MTX in MRP1- and MRP2-overexpressing Cells. The differential antifolate sensitivity profile shown in Table 1 suggested that alterations in cellular accumulation and antifolate polyglutamylation could play a role in explaining the marked level of drug resistance. To address this issue, we measured the accumulation of [3H]MTX and its conversion to polyglutamates after 4 and 24 h of exposure to 1 μM [3H]MTX in the absence or presence of 0.5 mM probenecid (Fig. 2). After 4 h of incubation with [3H]MTX, the total intracellular concentrations of [3H]MTX in 2008/MRP1 and 2008/MRP2 cells were 60 and 50% lower, respectively, than that in 2008 cells. Furthermore, following incubation for 24 h, the accumulation of [3H]MTX was significantly decreased in both 2008/MRP1 (3.3-fold) and 2008/MRP2 cells (2-fold) compared to 2008 cells (164 ± 39 pmol/10⁷ cells). Coincubations with 0.5 mM probenecid resulted in a 2-fold increase in the total [3H]MTX accumulation in 2008, 2008/MRP1, and 2008/MRP2 cells, both after 4 and 24 h of incubation with [3H]MTX.

Analysis of [3H]MTX-polyglutamate formation at each of the experimental conditions showed that the absolute numbers of long-chain MTX-polyglutamates (MTX-Glu4–6) formed after 4 and 24 h of incubation was 5-fold lower in 2008/MRP1 cells and 2–4-fold lower in 2008/MRP2 cells compared to parental 2008 cells. Following 24 h......
of exposure to $[^{3}H]$MTX in the presence of probenecid, both parental MRP1 and MRP2 transfectants had >70% of their MTX converted to long-chain polyglutamates.

Discussion

Over the past decade, multiple efflux routes for MTX have been identified and characterized based on differences in potency of a series of selected inhibitors (22–24). Identification of the molecular pathways responsible for these efflux routes has not been addressed successfully, but it recently received renewed interest after several reports revealed the molecular cloning of multiple members of the MRP family (1–3, 6, 7). Functional characterization of various MRP homologues revealed some common features with those of MTX efflux systems (14, 19, 24). Here, we report that MRP1 and MRP2 overexpression can confer tumor cell resistance to polyglutatable antifolate drugs such as MTX and novel antifolates such as ZD1694 (Tomudex. Raltitrexed), recently approved for use of treatment of colorectal cancer (25). Because MRP1 and possibly MRP2 appear to be expressed in many human cancers (6, 16, 18), more knowledge about a possible MRP-related resistance of tumor cells to antifolate treatment can, therefore, be of clinical relevance (26).

Several lines of evidence establish that MRP1 and MRP2 can transport MTX and that their overexpression can confer antifolate drug resistance: (a) $[^{3}H]$MTX uptake into inside-out vesicles of MRP1 and MRP2 2008 cell transfectants was ATP dependent. This MRP1-mediated MTX transport was abolished by the MRP1-specific monoclonal antibody, MIB6. (b) The profile of antifolate drug resistance was consistent with the efficiency of polyglutamylation; poor formation of long-chain polyglutamates during short-term exposure conferred drug resistance, whereas rapid formation of long-chain polyglutamates retained drug sensitivity. (c) Finally, probenecid, a potent inhibitor of MRP1 and MRP2 efflux activity almost completely reversed the antifolate resistance phenotype.

It is well known that impairment of antifolate polyglutamylation due to a decreased activity of FPGS can confer drug resistance following short-term as well as long-term exposure, in particular to those antifolates (e.g., ZD1694) that are dependent on polyglutamylation for their cytotoxic activity (8, 10, 11). The fact that no major differences in the IC$_{50}$ values of MTX, GW1843, and ZD1694 observed after 4- or 72-h exposure indicate that alterations in FPGS activity are not an underlying mechanism, explaining the resistance phenotype following short-term drug exposure. The other observation that 2008/MRP1 and 2008/MRP2 cells display resistance to a 4-h exposure to MTX and GW1843 and a 1-h exposure to ZD1694 (but not a 4-h exposure to ZD1694) suggests that the polyglutamate chain length is the critical determinant for the MRP-mediated antifolate efflux and the conferrance of the drug resistance phenotype. GW1843 is efficiently transported into cells by the RFC and is also an excellent substrate for FPGS but is not metabolized to polyglutamate forms that are longer than the diglutamate form (12). The marked resistance to short-term GW1843 exposure suggests that the diglutamate form may still be a substrate for MRP1 and MRP2 but that longer-chain polyamionic polyglutamates are poor efflux substrates of these ATP-driven exporters.

A detailed analysis of $[^{3}H]$MTX polyglutamylation in 2008, 2008/MRP1, and 2008/MRP2 cells revealed a profile that can explain MTX resistance during short-term exposure and maintenance of drug sensitivity during long-term exposure. After 4 h of MTX exposure, the total accumulation as well as long-chain MTX polyglutamate formation was markedly impaired in 2008/MRP1 and 2008/MRP2 cells. MRP-mediated efflux of MTX or short-chain polyglutamates during the washing procedure in drug-free medium may, then, confer drug resistance. Although the total level of free MTX and that of the long-chain MTX-polyglutamates remains lower in MRP containing transfectants than in the parental cells after 24 h of exposure, these levels should be high enough to exceed the DHFR binding capacity for MTX. Hence, DHFR will be inhibited (by MTX itself or by polyglutamate forms), resulting in a potent growth-inhibitory effect after 72 h of continuous exposure. Probenecid (21, 22) was able to increase the accumulation of MTX and long-chain polyglutamates in 2008/MRP1, 2008/MRP2, and parental 2008 cells. The latter obser-

<table>
<thead>
<tr>
<th>Antifolate</th>
<th>Incubation time (h)</th>
<th>2008 cells</th>
<th>2008/MRP1 cells</th>
<th>2008/MRP2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (nM)</td>
<td>IC$_{50}$ (nM)</td>
<td>RF (m)</td>
<td>IC$_{50}$ (nM)</td>
</tr>
<tr>
<td>MTX</td>
<td>72</td>
<td>13.8 ± 5.1 nM</td>
<td>25.7 ± 10.6 nM</td>
<td>1.9</td>
</tr>
<tr>
<td>GW1843</td>
<td>4</td>
<td>1.1 ± 0.2 nM</td>
<td>4.0 ± 0.3 nM</td>
<td>3.6</td>
</tr>
<tr>
<td>Trimetrexate</td>
<td>4</td>
<td>1.1 ± 0.2 nM</td>
<td>2.9 ± 0.9 nM</td>
<td>2.6</td>
</tr>
<tr>
<td>MTX</td>
<td>4</td>
<td>12.2 ± 3.6 nM</td>
<td>4.9 ± 1.4 nM</td>
<td>0.4</td>
</tr>
<tr>
<td>MTX + probenecid</td>
<td>4</td>
<td>12.9 ± 6.8 μM</td>
<td>&gt;1000 μM</td>
<td>&gt;77.5</td>
</tr>
<tr>
<td>MTX + probenecid</td>
<td>4</td>
<td>3.2 ± 1.1 μM</td>
<td>7.3 ± 3.1 μM</td>
<td>2.3</td>
</tr>
<tr>
<td>MTX</td>
<td>4</td>
<td>1.2 ± 0.5 μM</td>
<td>3.5 ± 1.4 μM</td>
<td>2.9</td>
</tr>
<tr>
<td>GW1843</td>
<td>4</td>
<td>1.6 ± 1.0 μM</td>
<td>&gt;250 μM</td>
<td>160.0 ± 32.0 μM</td>
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<tr>
<td>ZD1694</td>
<td>4</td>
<td>0.043 ± 0.011 μM</td>
<td>0.138 ± 0.076 μM</td>
<td>3.2</td>
</tr>
<tr>
<td>ZD1694</td>
<td>4</td>
<td>0.778 ± 0.326 μM</td>
<td>107 ± 31 μM</td>
<td>138.0</td>
</tr>
</tbody>
</table>

$^*$IC$_{50}$ values are means ± SD of four to six experiments.

$^b$RF, resistance factor, relative to 2008 cells.

$^c$Significantly different from parental: $P < 0.05$.

$^d$Probenecid (0.5 mM) present during 4-h drug exposure.

$^e$Probenecid (0.5 mM) present during 4-h drug exposure and during the drug-free period.

Fig. 2. $[^{3}H]$MTX accumulation and polyglutamylation in 2008, 2008/MRP1, and 2008/MRP2 cells. Monolayer cells were incubated with $[^{3}H]$MTX (1 μM) for 4 and 24 h, either in the absence or presence of 0.5 mM probenecid. ■, the total pool of long-chain polyglutamates of $[^{3}H]$MTX (MTX-Glu$_{1-4}$); □, the total pool of free MTX (MTX-Glu$_{1}$) and the short-chain MTX polyglutamates (MTX-Glu$_{1-3}$). Columns, means of three to four independent experiments; bars, SD.
viation may be explained by the fact that 2008 cells express some MRP1 (6). Our hypothesis is that the probenecid-dependent inhibition of MRP-mediated MTX efflux increases the levels of MTX long-chain polyglutamates in the MRP-expressing cells to such an extent (similar to the 4-h exposure of parental 2008 cells in the absence of probenecid; Fig 2) that it will result in long-term inhibition of DHFR and HIFR drug uptake after 4 h of drug incubation. Consequently, probenecid was able to reverse MTX resistance in 2008/MRP1 and 2008/MRP2 cells after short-term MTX exposure.

Our demonstration that MRP1 and MRP2 are involved in antifolate efflux raises the interesting possibility that MRPs may have a physiological role in controlling cellular reduced folate cofactor homeostasis. Indeed, two recent reports (27, 28) observed that the loss of a folate/MTX efflux pump in Chinese hamster ovary cells, highly resistant to the lipophilic antifolate DHFR inhibitor pyrimethamine, increased the intracellular folate pool by 3-fold. This expanded intracellular folate pool allowed these cells to bypass the folate-depleting effects of DHFR inhibitors, which could explain the 1000-fold resistance to pyrimethamine. Overexpression of folate efflux pumps could even lead to decreased intracellular folate pools, which is usually associated with collateral sensitivity to lipophilic DHFR inhibitors, a phenomenon that can also be observed in cells with defective RFC-mediated uptake of MTX/reduced folate co factors (29). Indirect evidence for decreased folate pools in 2008/MRP1 and 2008/MRP2 cells is suggested by their collateral sensitivity to the lipophilic DHFR inhibitor in vitro: a potentiating agent of L1210 tumor cell growth in vivo and in vivo: a new agent for clinical studies. Cancer Res., 57: 5579–5586, 1997.


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