ABC2 harboring the Gly482 mutation confers high-level resistance to various hydrophilic antifolates


Abstract

ABC2 is an ATP-binding cassette transporter that confers resistance to various chemotherapeutic agents. Recent studies have established that an Arg (wild-type) to Gly mutation at amino acid 482 in ABC2 alters substrate specificity. Here, we explored the role of this G482 mutation in antifolate resistance using a clinically relevant 4-hour drug exposure. Stable transfectants overexpressing the mutant G482 transporter displayed 120-, 1,000-, and >6,250-fold resistance to the antifolates methotrexate, GW1843, and Tomudex, respectively, relative to parental human embryonic kidney cells. Moreover, although overexpressing equal transporter levels at the plasma membrane, G482-ABC2 cells were 6-, 23-, and >521-fold more resistant to methotrexate, GW1843, and Tomudex, respectively, than R482-ABC2 cells. In contrast, upon a continuous (72-hour) drug exposure, both the G482- and R482-ABC2 cells lost almost all their antifolate resistance; this result was consistent with the inability of ABC2 to extrude long-chain antifolate polyglutamates. Ko143, a specific and potent ABC2 inhibitor reversed methotrexate resistance in both G482- and R482-ABC2 cells. Consistently, whereas the pool of free methotrexate in parental human embryonic kidney cells was prominent after 4 hours of transport with 1 μmol/L [3H]methotrexate, in R482- and G482-ABC2 cells, it was minimal. Furthermore, G482-ABC2 cells contained marked decreases in the di- and triglutamate species of [3H]methotrexate at 4 hours of incubation with methotrexate and in the tetra- and pentaglutamates at 24 hours. These changes were not associated with any significant decrease in polyglutamyl-γ-glutamate synthetase activity. These results provide the first evidence that the G482-ABC2 mutation confers high-level resistance to various hydrophilic antifolates. (Cancer Res 2005; 65(18): 8414-22)

Introduction

ATP-binding cassette (ABC) transporters constitute the largest superfamily of human cellular transporters currently containing 48 members classified in seven subfamilies termed A to G (1). ABC2, which is also known as the breast cancer resistance protein/mitoxantrone-resistance/ABC protein, is comprised of 655 amino acids, one transmembrane domain with six putative transmembrane segments, and a single ATP-binding site. Therefore, ABC2 is considered a “half-transporter” (2–4) like other members of the ABCG subfamily including the Drosophila white protein orthologue (5).

Overexpression of ABC2 has been shown to confer resistance to a variety of chemotherapeutic agents, albeit at different levels, including the anthracycline doxorubicin (11); and the antifolate methotrexate (12–14). During the development of a flow cytometric assay for the detection of ABC2 efflux activity, a disparity was noted in the outward transport of rhodamine 123 in ABC2-overexpressing tumor cell lines (15); sequence analysis of the ABC2 cDNAs isolated from the various cell lines identified two acquired mutations, G482 and T482. Introduction of the mutant G482- and T482-ABC2 genes into drug-sensitive cells resulted in altered ABC2 substrate specificity and major shifts in the cross-resistance profiles (16). Furthermore, a consistent pattern of R482 substitution was also described for the mouse Abcg2 gene (17). In this study, the authors used mouse fibroblasts lacking functional Mdr1a, Mdr1b, and multidrug resistance protein-1 (MRP1), which were selected in mitoxantrone, doxorubicin, and topotecan. A much higher level of resistance was observed in the doxorubicin-selected cells when compared with those selected in mitoxantrone and topotecan. Sequencing of the Abcg2 gene in these drug-resistant cell lines identified two mutations, R482M and R482S.

Whereas the wild-type (R482) ABC2 transported methotrexate and conferred a substantial level of resistance to this antifolate upon a continuous 7-day drug exposure in a mitoxantrone stepwise selected MCF-7/MX cell line, the mutant G482 failed to confer resistance to methotrexate under these conditions (13). Furthermore, the G482-ABC2 was apparently unable to transport methotrexate in membrane vesicles isolated from cells overexpressing this mutant transporter (18–20). In this respect, we have previously shown that cell lines with MRP1 (ABCC1) and MRP2 (ABCC2) overexpression display high-level resistance to short-term (4 hours) exposure to antifolates but retain parental sensitivity upon a continuous (72 hours) drug exposure (21). Adopting this experimental approach, we here explored the role of the G482-ABC2 mutation in cellular resistance to various antifolates as compared with the wild-type R482 counterpart and parental human embryonic kidney (HEK293) cells. We find that cells harboring G482-ABC2 overexpression display high-level resistance to various hydrophilic antifolates upon 4 hours of exposure, but retain sensitivity on continuous drug exposure. Furthermore, a marked decrease in the accumulation of methotrexate polyglutamates was found in G482- and R482-ABC2 cells. These results establish that the G482-ABC2 is a dominant gain of function mutation with regard to hydrophilic antifolate resistance.
Materials and Methods

Drugs and chemicals. Methotrexate, rhodamine 123, tetramethylrosamine, Triton X-100, Tween 20, poly-o-lysine (70,000-150,000 molecular weight) and ATP were obtained from Sigma Chemical Co. (St. Louis, MO). G-418 hydrochloride was from Calbiochem-Novabiochem, San Diego, CA. Mitoxantrone hydrochloride was from Cytosanum of Great Britain Ltd. (Gosport, Hampshire, England). Tomudex (Baltirexet, ZD1694) and GW1843 were generous gifts from AstraZeneca (Stanhope Gate, London, United Kingdom) and GlaxoSmithKline (Brentford, Middlesex, United Kingdom), respectively. Ko143 was kindly provided by Prof. A.H. Schinkel, Netherlands Cancer Institute, Amsterdam, the Netherlands. [3H]Methotrexate (19 Ci/μmol) was obtained from Moravek Biochemicals (Brea, CA) and purified prior to use as described previously (22). [2-3H]-Glutamic acid (NET-395) was purchased from New England Nuclear Life Science Products, Boston, MA.

Tissue culture. HEK293 cells and their stable transfectants over-expressing the R482- and G482-ABCG2 (16) were grown under monolayer conditions in RPMI 1640 (Biological Industries, Beth-Haemek, Israel) containing 10% FCS (TechnoGen, Gaithersburg, MD), 2 mmol/L glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin; the growth medium of the R482- and G482-ABCG2 transfectants was routinely supplemented with 2 mg/mL G-418. Upon multiple growth-inhibition experiments done with both the empty vector–transfected HEK293 cells that grow in G-418-containing medium as well as with the untransfected HEK293 cells we obtained superiorimposable drug sensitivities; thus, untransfected HEK293 cells were used as the parental cell line on subsequent experiments. The human ovarian carcinoma sublines 2008/MRP1, 2008/MRP2, and 2008/MRP3 which are stably transduced with MRPI, MRPII, and MRPIII cDNAs (kindly provided by Prof. P. Borst, The Netherlands Cancer Institute, Amsterdam, the Netherlands) were cultured in the above RPMI 1640. The Emt1 Chinese hamster ovary cell line was derived in our lab by stepwise selection in increasing emetine concentrations resulting in a stable MDR1 gene amplification and consequent P-glycoprotein (Pgp) overexpression (23). This cell line was routinely grown in RPMI 1640 supplemented with 1 μmol/L emetine.

Extraction of membrane proteins from cultured cells. Exponentially growing cells were detached by trypsinization, washed twice with PBS, and harvested by centrifugation. Cells (1 x 10^6) were then incubated in a lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 50 mmol/L 2-mercaptoethanol, 0.5% Triton X-100, and a Complete mini mixture of protease inhibitors (Roche Diagnostics GmbH, Penzberg, Germany) containing 10 μg/mL phenylmethylsulfonyl fluoride, 60 μg/mL aprotinin, 5 μg/mL leupeptin, 10 μg/mL pepstatin, 5 mmol/L EGTA (at pH 8), and 5 mmol/L EDTA (at pH 8). After 1 hour incubation on ice, the protein extract was centrifuged and aliquots of the supernatant were stored at −80°C until analysis. Protein content was determined using the Bio-Rad protein assay according to Bradford.

Western blot analysis of ABCG2, MRPI to MRPIII, and Pgp expression. To examine the expression of ABCG2, MRPI to MRPIII, and Pgp in the various cell lines, nonionic detergent-soluble proteins were extracted. Proteins (50 μg) were then resolved by electrophoresis on 7% polyacrylamide gels containing SDS and electroblotted onto Protran BA83 cellulose nitrate membranes (Schleicher & Schuell, Dassel, Germany). The blots were blocked for 1 hour at room temperature in TBS buffer (10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 0.05% Tween 20 containing 1% skim milk. The blots were then reacted with the following rabbit anti-human breast cancer resistance protein, MRPI, and Pgp monoclonal antibodies (generously provided by Prof. R.J. Schepers and Dr. G.L. Scheffer VL University Medical Center, Amsterdam, the Netherlands): rat anti-ABCG2 (BXP-53; at a dilution of 1:1,000), rat anti-MRP1 (MRP-rl; 1:1,000), mouse anti-MRP2 (M3-JL-6; 1:500), -MRPIII (M3-JL-21; 1:500), -MRPII (M3-I-8; 1:500), -MRPI (M3-JL-1; 1:750), and anti-Pgp (J533-1; 1:100). Blots were then rinsed thrice in the same buffer for 10 minutes each at room temperature, and reacted with second antibodies consisting of either horseradish peroxidase–conjugated goat anti-mouse or anti-rat IgG (1:10,000 dilution, Jackson ImmunoResearch Labs, West Grove, PA) for 1 hour at room temperature. Following three washes (each for 10 minutes) in TBS at room temperature, enhanced chemiluminescence detection was done according to the manufacturer’s instructions (Biological Industries). To normalize for loading differences, the nylon membranes were first stripped using the following procedure: the nylon membranes were incubated for 10 minutes in a stripping solution containing 0.5 mol/L Na2CO3, 0.5 mol/L acetic acid (pH 2.4). Then, the nylon membranes were washed twice with TBS and reacted with an antibody against β-tubulin (clone 2-28-33 from Sigma; 1:4,000).

Flow cytometric assay of cellular tetramethylrosamine accumulation. Cells (1 x 10^6) in growth medium containing 20 mmol/L HEPES (pH 7.3) were distributed into 15 mL test tubes. Then, tetramethylrosamine was added to the growth medium at various concentrations. After 30 minutes of incubation at 37°C, cells were transferred to ice and centrifuged at 4°C. Cells were then suspended in ice-cold RPMI 1640 containing 20 mmol/L HEPES (pH 7.3) and cellular fluorescence was determined with a flow cytometer. Excitation of tetramethylrosamine was at 550 nm and emission was collected at 574 nm.

Antifolate growth inhibition assay. Cells (8 x 10^5/L-1.2 x 10^6/well) were seeded in 24-well plates in growth medium. For the 4-hour drug exposure experiments, cells were then allowed to adhere to poly-o-lysine (0.1 mg/mL in PBS)-coated plates for 24 hours at 37°C. Attached cells were then exposed to various concentrations of methotrexate, Tomudex, or GW1843 for 4 hours at 37°C. Following which the drug-containing medium was aspirated and three careful washes with 10-minute intervals with 2.5 mL RPMI 1640 containing 2% FCS (Life Technologies) at 37°C were done. Drug-free medium was added (1 mL/well) and cultures were incubated for 3 days at 37°C. Then, cells were detached by trypsinization and the number of viable cells was determined by a hemocytometer after trypan blue staining.

Doxorubicin growth inhibition assay. Cells (8 x 10^5/L-1.2 x 10^6/well) were seeded in 24-well plates in growth medium (2 mL/well). Cells were allowed to adhere to the substrate for 24 hours at 37°C. Attached cells were then exposed to various concentrations of doxorubicin for 72 hours at 37°C. Finally, cells were detached by trypsinization and the number of viable cells was determined using a hemocytometer after trypan blue staining.

Assay of [3H]methotrexate transport and analysis of its polyglutamates. HEK293 cells and their stable R482- and G482-ABCG2 transfectants were cultured in 80 cm2 flasks until ~70% confluence was achieved. Cells were then incubated for 4 hours in growth medium containing 1 μmol/L [3H]methotrexate. Thereafter, cells were washed twice with 10 mL of ice-cold HEPES-buffered saline (HBS; pH 7.4), harvested by trypsinization, suspended in 10 mL ice-cold HBS, and centrifuged. Cell pellets were resuspended in 1 mL ice-cold HBS, 10 μL of which were used for cell counting. 100 μL for radioactivity determination, and 890 μL for [3H]methotrexate polyglutamate analysis by high-pressure liquid chromatography (HPLC) as previously described (21).

Isolation of plasma membrane vesicles and assay of ATP-dependent transport of [3H]methotrexate. The preparation of plasma membrane vesicles from HEK293 cells and their R482- and G482-ABCG2 transfectants was done in a hypotonic buffer followed by discontinuous sucrose gradient centrifugation as previously described (21). Measurement of the ATP-dependent transport of [3H]methotrexate was done in an ATP regeneration system containing creatine phosphate and creatine kinase as previously detailed (20).

Folylypoly-γ-glutamate synthetase activity assay. The catalytic activity of folylypoly-γ-glutamate synthetase (FPGS) in total cell extracts was determined by the incorporation of [2-3H]-γ-glutamic acid into methotrexate (14) followed by chromatography on Sep-Pak C18 cartridges (Millipore, Waters Associates, Etten-Leur, the Netherlands) as previously described (24).

ABCG2 sequence analysis. In order to confirm that the G482-ABCG2 transfectant harbored the correct mutation, DNA was first extracted from both the R482- and G482-ABCG2 transfectants. Then, genomic PCR of nearly the entire ABCG2 coding region was done using 10 pmol of each of overlapping primers in 2xReddyMix PCR master mix reaction buffer according to the instructions of the manufacturer (ABgene, Surrey, United Kingdom). The upstream and downstream ABCG2 primers that were designed using the Gene Runner Software version 3.0 were as follows: 1S,
in order to estimate the fraction of methotrexate that is bound to intracellular dihydrofolate reductase, cells were first incubated with \(1 \mu\text{mol/L} \ [3\text{H}]\text{methotrexate} \) for 30 and 60 minutes. Then, after reaching steady-state methotrexate levels, cells were washed thrice in drug-free medium at \(37^\circ\text{C}\) with 15-minute intervals (at \(37^\circ\text{C}\)) in between washes to allow the complete efflux of the free methotrexate. The residual cellular radioactivity represents the methotrexate fraction that is tightly bound to dihydrofolate reductase.

**Statistical analysis.** We used a paired Student's \(t\) test to examine the significance of the difference between two populations for a certain variable. A difference between the averages of two populations was considered significant if the \(P\) value obtained was <0.05.

**Scanning densitometry.** Relative ABCG2, MRP1 to MRP5, and Pgp protein levels were determined by scanning densitometry of several linear exposures obtained from multiple independent experiments using the program “TINA” (version 2.10g). The value obtained was divided by the densitometric value of \(\beta\)-tubulin.

### Results

**Expression of ABCG2 and various ABC transporters in parental HEK293 cells and their R482- and G482-ABCG2 transfectants.** Using genomic PCR and DNA sequencing, we first confirmed that the R482- and G482-ABCG2 transfectants did harbor these amino acids. Because ABCG2 and ABCB1 (MRP1) to ABCC5 (MRP5) can handle folates and antifolates as transport substrates, their expression status was determined by multiple Western blot analyses in parental HEK293 cells and their R482- and G482-ABCG2 transfectants (Fig. 1). ABCG2 was equally overexpressed in both R482- and G482 transfectants on nonlinear and linear film exposures, but was not detectable in HEK293 cells (Fig. 1A and 1B). Furthermore, MRP1 (Fig. 1D), MRP4 (Fig. 1F), and MRP5 (Fig. 1L) were equally expressed, albeit at low levels, in HEK293, R482-, and G482-ABCG2 cells. In contrast, none of these cell lines expressed any detectable levels of MRP2 (Fig. 1F), MRP3 (Fig. 1H), and Pgp (ABCB1; Fig. 1V). Reprobing the individual blots with a monoclonal antibody to \(\beta\)-tubulin confirmed that equal amounts of membrane proteins were being analyzed (Fig. 1C, E, G, I, K, M, and O).

**Subcellular localization of ABCG2 in the plasma membrane of R482-, G482-, and T482-ABCG2 transfectants.** We undertook an immunohistochemistry analysis in order to determine whether the overexpressed ABCG2 was properly sorted out to the plasma membrane in the various transfectants. Indeed, using a monoclonal antibody to ABCG2, it was found that ABCG2 was highly and equally localized to the plasma membrane of R482-, G482-, and the T482-ABCG2 transfectants but was undetectable in their parental HEK293 cells (data not shown).

**Doxorubicin resistance and tetramethylrosamine accumulation in the R482- and G482-ABCG2 transfectants.** Recent studies have shown that ABCG2 harboring a G482 mutation displays high-level resistance to doxorubicin and could thereby be distinguished from its wild-type counterpart (R482-ABCG2) that exhibits a low level of resistance to this anthracycline (16). Consistently, the mutant G482-ABCG2 transfectant was 96-fold more resistant to doxorubicin, whereas the R482-ABCG2 transfectant displayed only 4-fold resistance, relative to parental HEK293 cells (Table 1B). Furthermore, both the G482- and R482-ABCG2 cells essentially resumed parental cell sensitivity to doxorubicin upon treatment with Ko143 (Table 1B), a specific and potent inhibitor of ABCG2 transport (25). These results confirm that the mutant G482-ABCG2 mediates high-level resistance to doxorubicin.

![Western blot analysis of ABCG2, MRP1 to MRP5, and Pgp in parental HEK293 cells and their R482- and G482-ABCG2 transfectants.](image-url)
order to provide direct evidence for the distinct efflux capabilities of the R482- and G482-ABCG2 transporters, the chromophore efflux activities of these transfectants were explored using 30 minutes accumulation of tetramethylrosamine, a rhodamine 123 analogue. Parental HEK293 cells and their R482-ABCG2 overexpressing transfectant displayed a similar dose-dependent accumulation of tetramethylrosamine (Fig. 2A). In contrast, G482-ABCG2 cells exhibited a complete exclusion of tetramethylrosamine. After exploring the differential exclusion of tetramethylrosamine in the G482-ABCG2 transfectant, we examined tetramethylrosamine accumulation in the absence (Fig. 2C) or presence (Fig. 2D) of the ABCG2 transport inhibitor Ko143. In the absence of tetramethylrosamine, all cell lines displayed an identical background autofluorescence (Fig. 2B). Following 30 minutes of incubation with 100 nmol/L tetramethylrosamine, R482-ABCG2 cells and their parental HEK293 counterpart displayed a superimposable, high-level fluorescence, whereas G482-ABCG2 cells accumulated <1% of parental HEK293 cells’ fluorescence (Fig. 2C). Furthermore, in the presence of Ko143, cellular tetramethylrosamine fluorescence in G482-ABCG2 cells increased by almost 100-fold, thereby approximating the fluorescence level of both parental and R482-ABCG2 cells (Fig. 2D). In contrast, Ko143 had no effect on tetramethylrosamine accumulation in parental cells and the R482-ABCG2 transfectants. These results establish that unlike the wild-type R482 transporter, the mutant G482-ABCG2 is an efficient efflux transporter of tetramethylrosamine that can be potently inhibited by Ko143.

**G482-ABCG2 confers high-level resistance to various antifolates.** Because Hooijberg et al. (21) have originally shown that cell resistance to various antifolates only on a short-term (4 hours) drug exposure, we also explored the impact of G482-ABCG2 transfection on methotrexate resistance using a 4-hour drug exposure (Table 1A). R482-ABCG2 cells displayed 19-fold resistance to methotrexate relative to parental HEK293 cells, whereas G482-ABCG2 cells exhibited 120-fold resistance (Fig. 3A). Furthermore, Ko143 induced a nearly complete reversal of methotrexate resistance in both the R482- and G482-ABCG2 cells (Fig. 3B). Although, like its R482 and G482 counterparts, the T482-ABCG2 transfectant (16) overexpressed equal ABCG2 levels at the plasma membrane, it displayed only 10-fold resistance to methotrexate relative to parental HEK293 cells (Fig. 3C). Hence, in contrast to the markedly increased resistance to methotrexate in the G482-ABCG2 transfectant, substitution of the native R482 by T482 resulted in a decreased resistance to methotrexate. We next studied the effect of continuous drug exposure on methotrexate sensitivity. Remarkably, upon a 72-hour exposure to methotrexate (Table 1B), R482-ABCG2 cells resumed parental sensitivity to methotrexate, whereas the G482-ABCG2 cells retained only a residual resistance (3.3-fold; Fig. 3D).

The high-level resistance to methotrexate in the G482-ABCG2 cells prompted us to further explore the pattern of resistance to other hydrophilic antifolates including Tomudex (Raltitrexed) and GW1843. Upon 4-hour drug exposure (Table 1A), R482-ABCG2 cells displayed a moderate resistance to Tomudex (12-fold) relative to parental HEK293 cells, whereas G482-ABCG2 cells exhibited an extremely high-level resistance of >6,250-fold (Fig. 4A). Furthermore, whereas R482-ABCG2 cells showed 43-fold resistance to GW1843 relative to parental HEK293 cells, the mutant G482-ABCG2 cells were 1,000-fold resistant (Fig. 4C). Remarkably, upon a continuous (72 hours) exposure to Tomudex (Fig. 4B) and GW1843 (Fig. 4D), both the R482- and G482-ABCG2 cells retained only a residual resistance (~3-fold) to these antifolates (Table 1B).

**Decreased accumulation of [3H]methotrexate in the R482- and G482-ABCG2 transfectants.** Because the R482- and

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### Table 1. Summary of antifolate growth inhibition in the presence or absence of Ko143

**(A) 4-hour exposure**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methotrexate</td>
</tr>
<tr>
<td></td>
<td>−Ko143</td>
</tr>
<tr>
<td>HEK293</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>HEK293/R482-ABCG2</td>
<td>74 ± 17 (19)</td>
</tr>
<tr>
<td>HEK293/G482-ABCG2</td>
<td>466 ± 88 (120)</td>
</tr>
</tbody>
</table>

**(B) 72-hour exposure**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nmol/L)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Doxorubicin</td>
</tr>
<tr>
<td></td>
<td>−Ko143</td>
</tr>
<tr>
<td>HEK293</td>
<td>8.8 ± 4.9</td>
</tr>
<tr>
<td>HEK293/R482-ABCG2</td>
<td>38.5 ± 7.0 (4.4)</td>
</tr>
<tr>
<td>HEK293/G482-ABCG2</td>
<td>844 ± 319 (95.9)</td>
</tr>
</tbody>
</table>

NOTE: Fold resistance values are shown in parentheses.
G482-ABCG2 transfectants displayed a marked level of resistance to methotrexate and other antifolates. [3H]metothrexate accumulation was determined. Following 4 hours (Fig. 5A) and 24 hours (Fig. 5B) of incubation with 1 μmol/L [3H]metothrexate at 37°C, R482- and G482-ABCG2 cells showed a 2-fold decrease in [3H]metothrexate accumulation, relative to parental cells; this decreased accumulation of [3H]metothrexate di- and triglutamates was clearly reflected at 24 hours of incubation with [3H]metothrexate; G482-ABCG2 cells contained 6.5-fold and 4.5-fold decreased levels of tetra- and pentaglutamates of metothrexate, respectively, than their parental HEK293 cells (Table 2). Furthermore, this markedly decreased accumulation of metothrexate di- and triglutamates was confirmed by HPLC after 4 and 24 hours of incubation with 1 μmol/L [3H]metothrexate. After 4 hours of incubation, G482- and R482-ABCG2 cells contained ~3-fold and ~2-fold less di- and triglutamates of metothrexate, respectively, than their parental HEK293 cells (Table 2). Moreover, this markedly decreased accumulation of metothrexate di- to pentaglutamates in the G482- and R482-ABCG2 cells was not associated with any significant decrease in FPGS activity in R482- and G482-ABCG2 transfectants; the specific activities of FPGS in HEK293, R482- and G482-ABCG2 cells were respectively: 899 ± 163, 716 ± 131, and 708 ± 76 pmol [3H]glutamate incorporated/h/mg protein. Moreover, the specific activities of FPGS in R482- and G482-ABCG2 transfectants were also similar to those found in human leukemia CCRF-CEM cells (785 ± 75 pmol [3H]glutamate incorporated/h/mg protein), an established cell line that is known to well express folate/antifolate polyglutamylation activity (27). In contrast, CCRF-CEM/R30dm, an established FPGS-defective cell line (26) displayed <0.1% of the FPGS activity observed in HEK293 cells.

[3H]Methotrexate transport into isolated plasma membrane vesicles from R482- and G482-ABCG2 transfectants. Following the isolation of plasma membrane vesicles from R482- and G482-ABCG2 transfectants, as well as from their parental HEK293 cells, the ATP-dependent transport of [3H]methotrexate was determined. Thus, R482-ABCG2–overexpressing cells had an apparent Vmax of 1.28 ± 0.35 nmol/mg protein/min. Whereas, for some yet undefined basis, G482-ABCG2, as well as parental HEK293 cells, failed to show any measurable methotrexate transport activity that would be above the background level (<40 pmol/mg protein/min) obtained in the absence of exogenous MgATP. These results confirm previous reports which also studied [3H]methotrexate transport into isolated membrane vesicles from human cells overexpressing these ABCG2 variants (18, 20).

Discussion

Several lines of evidence suggest that ABCG2 harboring a G482 mutation is a dominant factor of resistance to various hydrophilic antifolates including methotrexate. First, cells overexpressing this mutant efflux transporter at the plasma membrane displayed striking resistance levels of 120-, 1,000-, and 6,250-fold to various folic acid antagonists including methotrexate, GW1843, and
Tomudex (Raltitrexed) upon a 4-hour drug exposure. Inasmuch as methotrexate and Tomudex are currently used in the treatment of various human cancers including acute lymphoblastic leukemia, breast cancer, osteosarcoma and colorectal cancer, these antifolates are administered as short-pulse infusions (i.e., bolus; refs. 28, 29). Therefore, the 4-hour drug exposure used here is representative of the clinical setting when compared with the continuous 7-day drug exposure used in cytotoxicity assays with cells overexpressing ABCG2 (13). Use of the 4-hour pulse exposure to antifolates is well established, particularly when assessing the role of multidrug resistance efflux transporters in methotrexate resistance (21). In this respect, Hooijberg et al. (21) first established the time-frame of 4 hours of methotrexate exposure and its importance in the detection of antifolate-resistance mediated by MRP1 and MRP2. Recently, it was shown that MRP5 consistently confers high-level resistance against methotrexate, GW1843, and Tomudex upon 4 hours of antifolate exposure (30). Hence, the use of a 4-hour drug exposure is consistent with both the bolus administration of methotrexate and its pharmacokinetic properties. For instance, high-dose methotrexate treatment is given in osteosarcoma patients at 12 g/m² as an i.v. bolus over 4 hours (31). Furthermore, the pharmacokinetic characteristics of methotrexate are also compatible with the 4-hour drug exposure; maximal methotrexate plasma concentrations ($C_{\text{max}}$) can be obtained as...
suggest that these transfectants extrude much of the free methotrexate pool of only 0.6 to 2.4 pmol/10^7 cells. These results G482 and R482-ABCG2 transfectants possessed a minimal free A incubated in growth medium containing 1 **,**, P < 0.001.; 0.05; **, P < 0.001.

Figure 5. Transport of [3H]methotrexate into HEK293 cells and their R482- and G482-ABCG2 transfectants. Cells growing in T75 tissue culture flasks were inoculated in growth medium containing 1 pmol/L [3H]methotrexate for 4 hours (A) or 24 hours (B). Cells were then washed twice with ice-cold HBS, detached by trypsinization, counted, lysed, and total cellular radioactivity was determined using scintillation counting. Columns, means; bars, ± SD from four independent experiments. Statistical significance relative to parental HEK293 cells: *, P < 0.05; **, P < 0.001.

fast as 0.5 to 1 hour after i.m. drug injection. Methotrexate elimination is also rapid and is triphasic; the first half-life is ~45 minutes and reflects the drug distribution phase, whereas the second half-life is about 3.5 hours and is primarily due to renal clearance. The terminal half-life is about 10 to 12 hours and may reflect enterohepatic circulation of methotrexate. Second, the high-level resistance to methotrexate (and doxorubicin) in these G482-ABCG2-overexpressing cells was completely reversed by the potent and specific ABCG2 inhibitor, Ko143 (25), thereby establishing that antifolate (and anthracycline) drug resistance is mediated by ABCG2. Third, whereas parental HEK293 cells had a substantial pool of free methotrexate of 10.9 pmol/10^7 cells, the G482 and R482-ABCG2 transfectants possessed a minimal free methotrexate pool of only 0.6 to 2.4 pmol/10^7 cells. These results suggest that these transfectants extrude much of the free intracellular methotrexate in an ABCG2-dependent manner. Fourth, G482- and R482-ABCG2–overexpressing cells contained a marked depletion of the di- and triglutamate species of methotrexate after 4 hours as well as tetra- and pentaglutamates after 24 hours of incubation with [3H]methotrexate; these results strongly suggest that the G482- and R482-ABCG2 interfere with the intracellular accumulation of antifolate polyglutamates.

Although cells overexpressing the R482- and G482-ABCG2 showed resistance to various chemotherapeutic drugs on continuous drug exposure (16), we show here that these cells were resistant to methotrexate and other antifolates only after 4 hours of drug exposure but lost most of this resistance after continuous (72-hour) drug exposure. Our results are consistent with the original finding of Hooijberg et al., (21) that ovarian carcinoma cells stably overexpressing MRP1 and MRP2 were >78- and >156-fold resistant to methotrexate and GW1843, respectively, but retained wild-type sensitivity after a continuous (72-hour) antifolate exposure. Thus, this group suggested that the mechanism underlying the loss of antifolate resistance after continuous drug exposure is that unlike various anticancer drugs, antifolates are retained within cells by a metabolic process known as polyglutamylation via the enzyme FPGS (26, 27). Because, unlike the MRPs, ABCG2 has the capacity to extrude mono-, di-, and triglutamates of methotrexate (18, 19), the longer-chain (i.e., >3 glutamate derivatives) polyglutamate derivatives of methotrexate and other polyglutamatable antifolates should be better retained in these cells, thereby resulting in restoration of parental drug sensitivity. Furthermore, as cellular synthesis and accumulation of methotrexate polyglutamates is a relatively slow process (27), during the 4-hour antifolate exposure most of the intracellular methotrexate is present in the mono-, di-, and triglutamate forms, as can be seen with parental HEK293 cells (Table 2). As such, ABCG2 which can transport mono-, di-, and triglutamate conjugates of methotrexate (18, 19) presumably extrudes these cytotoxic antifolate conjugates thereby adding to the resistance conferred by the efflux of the parent unconjugated drug.

Although we find that overexpression of the mutant G482- ABCG2 confers an extremely high level of resistance to various antifolates, several studies employing vesicles isolated from ABCG2-overexpressing cell lines (mostly HEK293 and also MCF-7

### Table 2. Analysis of methotrexate polyglutamates in G482- and R482-ABCG2 and their parental HEK293 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Time (h)</th>
<th>pmol/10 million cells</th>
<th>Total MTX</th>
<th>MTXglu1</th>
<th>MTXglu2</th>
<th>MTXglu3</th>
<th>MTXglu4</th>
<th>MTXglu5</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK/wild-type</td>
<td>4</td>
<td>20.8 ± 6.3 (0.009)</td>
<td>6.8 ± 1.7</td>
<td>9.0 ± 3.6</td>
<td>3.9 ± 2.1</td>
<td>0.7 ± 1.0</td>
<td>0.5 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>(P value)</td>
<td></td>
<td></td>
<td>9.9 ± 3.1 (0.009)</td>
<td>3.7 ± 2.0 (0.028)</td>
<td>4.3 ± 1.8 (0.030)</td>
<td>1.7 ± 0.8 (NS)</td>
<td>0.1 ± 0.3 (ND)</td>
<td>0.1 ± 0.2 (ND)</td>
</tr>
<tr>
<td>HEK/BCRP-G482</td>
<td>4</td>
<td>117 ± 3.1 (0.021)</td>
<td>7.1 ± 2.2 (NS)</td>
<td>2.9 ± 1.7 (0.009)</td>
<td>1.3 ± 1.0 (0.038)</td>
<td>0.3 ± 0.5 (ND)</td>
<td>0.1 ± 0.2 (ND)</td>
<td></td>
</tr>
<tr>
<td>(P value)</td>
<td></td>
<td></td>
<td>56.4 ± 4.2</td>
<td>7.6 ± 0.8</td>
<td>12.3 ± 0.8</td>
<td>21.6 ± 2.2</td>
<td>9.6 ± 1.1</td>
<td>5.3 ± 1.6</td>
</tr>
<tr>
<td>HEK/BCRP-R482</td>
<td>24</td>
<td>29.0 ± 3.5 (&lt;0.001)</td>
<td>7.5 ± 2.9 (NS)</td>
<td>8.4 ± 1.3 (0.001)</td>
<td>9.1 ± 3.3 (&lt;0.001)</td>
<td>2.9 ± 2.6 (0.005)</td>
<td>1.2 ± 1.1 (0.011)</td>
<td></td>
</tr>
<tr>
<td>(P value)</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>12.1 ± 1.9 (NS)</td>
<td>9.0 ± 2.6 (0.052)</td>
<td>5.2 ± 2.8 (&lt;0.001)</td>
<td>1.5 ± 1.2 (&lt;0.001)</td>
<td>1.0 ± 0.7 (0.002)</td>
</tr>
</tbody>
</table>

NOTE: NS, not significant; ND, not detectable.
and S1 cells) have recently suggested that R482-ABCG2 is capable of transporting methotrexate, whereas the G482-ABCG2 apparently lacks this capacity (18–20); our present [3H]methotrexate transport measurement with isolated plasma membrane vesicles from the various ABCG2-overexpressing variants corroborate these studies. In contrast to these findings, Janviliisir and his colleagues (32) have recently shown that the transport of the ABCG2 substrate Hoechst 33342 by G482-ABCG2 can be blocked by as low as 25 μmol/L methotrexate. Hence, this latter report can possibly support the suggestion that G482-ABCG2 may have the facility to accommodate and presumably transport methotrexate and other antifolates as well as their short-chain polyglutamate derivatives. It should be further noted that in none of the above studies (18–20) were G482-ABCG2 vesicles reported to transport any established substrate drug including mitoxantrone, doxorubicin, and epirubicin (16, 32).

The possibility exists that the loss of the positive (R) charge in the mutant G482 may differentially disrupt transporter dimerization during vesicle preparation; this could possibly occur either due to the hypotonic buffer conditions (33) or the nitrogen cavitation (18, 19) procedure used during the isolation of vesicles. In addition, upon cell lysis during vesicle preparation, the G482-ABCG2 (but not the wild-type R482) may potentially undergo some differential (non)physiologic posttranslational modification (e.g., phosphorylation or dephosphorylation) that may disrupt its transport capability. Clearly, further studies including functional reconstitution of G482-ABCG2 in proteoliposomes are necessary to show the direct ability of the G482-ABCG2 to transport methotrexate as well as other antifolates and their polyglutamate derivatives.

In the present report, we found that overexpression of the mutant G482-ABCG2 resulted in up to 6,250-fold antifolate resistance after 4 hours of drug exposure. However, this was associated with only 2-fold decrease in methotrexate monoglutamate levels. Although this may seem to be a discrepancy, it could be easily reconciled when considering the following finding. Because we have shown here that a significant fraction of intracellular methotrexate is tightly bound to its high affinity (Kd = 5 pmol/L) target enzyme, dihydrofolate reductase, the limited amount of intracellular free methotrexate (34) is either available for extrusion by the overexpressed ABCG2 and/or for polyglutamylation. Hence, it is not surprising that there was only a 2-fold reduction in the accumulation of the monoglutamate form of methotrexate after 4 hours of exposure. This finding is very consistent with our previous studies in which we also detected a 2-fold reduction in methotrexate monoglutamate levels in 2008 ovarian carcinoma cells with MRP1, MRP2 (21), and MRP3 overexpression (35).

In the current report, we found a prominent decrease in the accumulation of di- and triglutamates of methotrexate after 4 hours of incubation with methotrexate, and a striking decrease (>6-fold) in the accumulation of tetra- and pentaglutamates after 24 hours of incubation, particularly with the G482-ABCG2 transfectant. Hence, after 24 hours of incubation, the lack of sufficient substrate precursors (i.e., di- and triglutamates) for long-chain polyglutamylamion resulted in a drastic decrease (>6-fold) in the accumulation of tetra- and pentaglutamates of methotrexate. Our present results suggest that G482-ABCG2 efflux activity is an important factor which severely limits the accumulation of diglutamates to pentaglutamates of methotrexate. These findings are in accord with our previous analysis of methotrexate polyglutamylation in cells overexpressing MRP1 (21) and MRP3 (35), both of which displayed high-level resistance to methotrexate on short-term drug exposure; this was consistently associated with a 6-fold decrease in the long-chain polyglutamates of methotrexate (i.e., tetra- to hexaglutamates) after 24 hours of incubation with this drug. Remarkably, the 4- and 24-hour accumulation of methotrexate monoglutamates decreased by only 1.7- to 2.6-fold. Hence, as with MRP1 and MRP3, and based on the strong dependence of Tomudex on polyglutamylation for its pharmacologic activity (6,250-fold resistance in G482-ABCG2 cells), it seems that the ABCG2-dependent disruption of the accumulation of long-chain polyglutamate conjugates is a key determinant of antifolate resistance after clinically relevant short-term drug exposure.

Various mitoxantrone-selected cell lines, including MCF-7/MR cells with R482-ABCG2 overexpression, were shown to display high level resistance to continuous exposure to methotrexate (12, 13). This suggested that ABCG2 mediates the resistance to methotrexate. Surprisingly, however, after transfection with the R482- and G482-ABCG2, the same MCF-7 cells failed to show any significant methotrexate resistance despite the high level of ABCG2 overexpression. Hence, the lack of methotrexate resistance in R482- and G482- overexpressing ABCG2 transfectants may possibly reflect a higher proportion of cytoplasmic targeting rather than plasma membrane localization of ABCG2. In fact, in the above studies, no documentation of the plasma membrane localization of ABCG2 was explored, thereby leaving the possibility that ABCG2 may be significantly localized to the cytoplasm (i.e., in the ER compartment). This may be supported by recent studies showing that various conditions can bring about a rapid cytoplasmic localization of ABCG2 (36, 37). In contrast, in the current report, equal transporter overexpression was present in the plasma membrane of the R482- and G482-ABCG2 transfectants as revealed by immunohistochemistry.

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References


G482 BCRP and Antifolate Resistance
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