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ORIGINAL ARTICLE

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Mutant Gly482 and Thr482 ABCG2 mediate high-level resistance to lipophilic antifolates

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Abstract Cellular uptake of hydrophilic antifolates proceeds via the reduced folate carrier whereas lipophilic antifolates enter cells by diffusion. Recently we have shown that transfectant cells overexpressing the mutant G482 ABCG2 displayed 120–6,250-fold resistance to hydrophilic antifolates than untransfected cells upon 4 h drug exposure, but lost almost all their antifolate resistance upon 72 h drug exposure (Shafran et al. in Cancer Res 65:8414–8422, 2005). Here we explored the ability of the wild type (WT) R482—as well as the mutant G482—and T482 ABCG2 to confer resistance to lipophilic antifolate inhibitors of dihydrofolate reductase (trimetrexate, piritrexim, metoprine and pyrimethamine) and thymidylate synthase (AG337, AG377 and AG331). Lipophilic antifolate resistance was determined using growth inhibition assays upon 72 h drug exposure. Cells overexpressing these mutant efflux transporters displayed up to 106-fold resistance to lipophilic antifolates relative to untransfected cells; this resistance was reversed by the specific and potent ABCG2 efflux inhibitor Ko143. In contrast, cells overexpressing the WT R482 ABCG2 exhibited either no or only a low-level of lipophilic antifolate resistance. These results provide the first evidence that overexpression of the mutant G482- and T482 but not the WT R482 ABCG2 confers a high-level of resistance to lipophilic antifolates. The high membrane partitioning of lipophilic antifolates along with the large confinement of ABCG2 to the plasma membrane suggest that these mutant ABCG2 transporters may possibly recognize and extrude lipophilic antifolates from the lipid bilayer. The potential implications to cancer chemotherapy as well as the mechanism of anticancer drug extrusion by these mutant exporters are discussed.

Keywords ABC transporter · Chemotherapy · Lipophilic antifolates · Efflux · Drug resistance

Abbreviations ABC: ATP-binding cassette protein · BCRP: Breast cancer resistance protein · MRP: Multidrug resistance protein · Pgp: P-glycoprotein · DHFR: Dihydrofolate reductase · TS: Thymidylate synthase

Introduction

The ATP-binding cassette (ABC) proteins are expressed in all living organisms and harbor two relatively conserved structural elements [1, 2]; the first known as ABC is a conserved ATP-binding domain whereas the second comprises conserved hydrophobic transmembrane domains (TMDs). Active ABC proteins consist of at least two TMDs and two ABCs; these domains may be contained within one polypeptide chain (i.e., full transporters) as with the multidrug resistance (MDR) efflux transporters P-glycoprotein (Pgp, ABCB1) [3] and MDR protein 1 (MRP1, ABCC1) [4–6] or may be present within two separate proteins (so called 'half-transporters') as with another MDR efflux transporter, breast cancer resistance protein (BCRP/ABCG2) [7–11]. In the latter case, homodimerization of these half-transporters appears to be a prerequisite for this ABC transporter to become functional [12–14]. The TMDs contain polypeptide chains spanning the membrane multiple times, typically forming six hydrophobic transmembrane α-helices in each TMD. The putative conformational changes within these TMDs are believed to play a pivotal role in the transport of various substrate molecules through these transporters. The cytoplasmic ABC domains are the site of ATP-binding and hydrolysis, thereby providing the energy for the uphill transport of

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various substrates. Importantly, there is a tight coupling between substrate translocation and ATP hydrolysis; it is believed that the interaction of these ABC domains with the TMDs provides the transmission gear connecting substrate binding and its translocation across biomembranes to ATP hydrolysis.

Currently, 49 ABC genes have been identified in humans including the multidrug transporters which are plasma membrane glycoproteins conferring resistance to various chemotherapeutic agents in different malignant cells. Physiologically, these ABC transporters play an important protective role against multiple toxicants in a variety of tissues, especially in secretory and absorptive epithelia as well as in blood–tissue barriers including the blood–brain barrier. The three major MDR ABC proteins are MDR1 (Pgp, ABCB1) [3], MRP1 (ABCC1) [4–6] as well as ABCG2 (BCRP/ABCP/MXR) [1, 2, 7–11]. Pgp and MRP1 transport a large array of structurally unrelated hydrophobic cytotoxic drugs and in addition MRP1 can also extrude anionic drugs and anionic conjugates. Similarly, the transport properties of ABCG2 were found to be overlapping those of Pgp and MRPs, the latter of which currently encompass nine members including MRP1 through MRP7 as well as ABCC11 and ABCC12 [5, 6]. Hence these three primary groups of MDR efflux transporters including MRPs, Pgp and ABCG2 form a unique defense network against multiple chemotherapeutic agents and cellular toxicants.

Recently we have shown that transfectant cells overexpressing the mutant G482 ABCG2 display 120–6,250-fold resistance to pulse exposure (4 h) to various hydrophilic antifolates including methotrexate (MTX), raltitrexed (Tomudex) and GW1843 [15]. These hydrophilic antifolates depend on the reduced folate carrier (RFC) for their cellular entry as well as on folylpoly-γ-glutamate synthetase (FPGS) activity for their cellular retention via conversion to long-chain polyglutamate conjugates. This marked hydrophilic antifolate resistance was reversed by the potent and specific ABCG2 efflux inhibitor Ko143 [16]. In contrast, lipophilic antifolates enter cells by diffusion and are therefore independent of the RFC for their uptake [17, 18]. Indeed, lipophilic antifolates were rationally designed to circumvent some major mechanisms of hydrophilic antifolate (e.g., MTX) resistance including transport-related alterations and decreased FPGS-based resistance phenomena [19, 20]. Specifically, lipophilic antifolates overcome various modalities of hydrophilic antifolate resistance that are associated with loss of function of the RFC [21] as well as decreased FPGS activity [22]. For example, trimetrexate is a liphophilic antifolate inhibitor of dihydrofolate reductase (DHFR) that has been introduced into the clinic in order to circumvent antifolate resistance that is due to the loss of RFC and/or FPGS activity (for review see [23]). Likewise, AG337 (Thymitaq, Nolatrexed), a lipophilic antifolate inhibitor of thymidylate synthase (TS) has proven clinical activity against various human cancers (for review see [24]). Consistently, various hydrophilic antifolate-resistant cell lines with loss of function of the RFC and/or FPGS display a marked hypersensitivity to trimetrexate, AG337 and AG377 [21, 22].

In order to identify cellular determinants contributing to lipophilic antifolate resistance, we here explored the ability of the wild type (WT) (R482) and mutant G482- and T482 ABCG2 to confer resistance to a continuous (72 h) exposure to various lipophilic antifolates. The latter including structural analogues of MTX and small 2,4-diaminopyrimidines (Fig. 1) enter cells by diffusion and are therefore independent of the RFC for their uptake [17, 18, 23, 24]; these lipid-soluble antifolates also lack a glutamate side chain and thus cannot undergo polyglutamylation. We find here that cells overexpressing the mutant G482- and T482 ABCG2 transporters display high-level resistance to various lipophilic antifolates; this resistance was reversed by the ABCG2 efflux inhibitor Ko143. In contrast, cells overexpressing the WT R482 ABCG2 exhibited low-level resistance to lipophilic antifolates. These results suggest that mutant G482- and T482 ABCG2 recognize both water-soluble and lipid-soluble antifolates. Moreover, unlike hydrophilic antifolates, these mutant ABCG2 transporters confer resistance to a continuous exposure to clinically active lipophilic antifolates. Hence based upon the high membrane co-partitioning of lipophilic antifolates along with the large confinement of ABCG2 to the plasma membrane we here propose that G482- and T482 ABCG2 may possibly recognize and extrude lipophilic antifolates from the lipid bilayer into the extracellular milieu.

Materials and methods

Drugs and chemicals Pyrimethamine, doxorubicin, paclitaxel, cisplatinum, tetramethylrosamine and poly-D-lysine (70,000–150,000 MW) were obtained from Sigma Chemical Co. (St. Louis, MO). Trimetrexate gluconate was originally provided by Dr D. Fry, Warner Lambert/Park Arbor, Ann Arbor, MI. Piritrexim isethionate and metoprine were generous gifts from Dr N. Clendeninn, GlaxoSmithKline, Research Triangle Park, Chapel Hill, NC. AG337 (Thymitaq, Nolatrexed), AG331 and AG377 were kindly provided by Dr T.J. Boritzki, Agouron Pharmaceuticals, San Diego, CA. G-418 hydrochloride was from Calbiochem–Novabiochem, San Diego, CA. Ko143 was kindly provided by Prof. A.H. Schinkel, Netherlands Cancer Institute, Amsterdam, The Netherlands.

Tissue culture Human embryonic kidney cells (HEK293) and their stable transfectants overexpressing the WT R482-, as well as the mutant G482- and T482 ABCG2 (kindly provided by Dr S.E. Bates) [25] were grown under monolayer conditions in RPMI-1640 medium (GIBCO) containing 10% fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin (Biological Industries, Beth-Haemek, Israel); the growth medium of the R482, G482- and T482 ABCG2 transfectants was routinely supplemented with 2 mg/ml G-418.

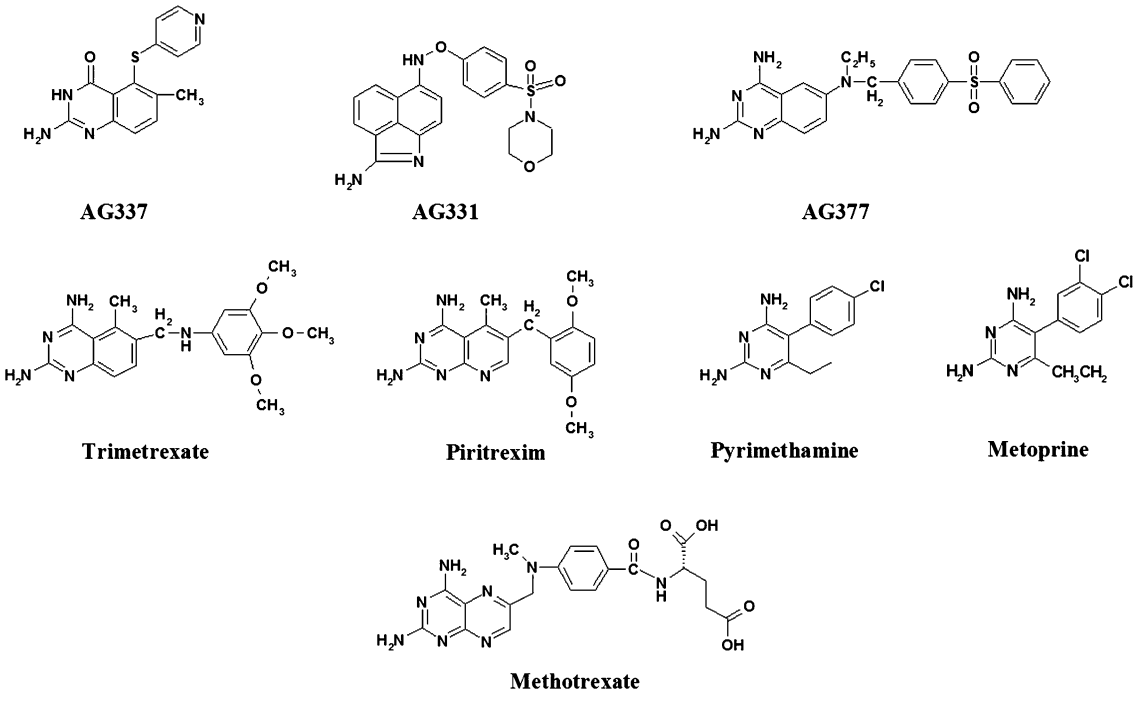

Fig. 1 Structural formulas of MTX and various lipophilic antifolate inhibitors of dihydrofolate reductase and thymidylate synthase

Western blot analysis of ABCG2 expression To examine the expression of ABCG2 in the various cell lines, non-ionic detergent-soluble proteins were extracted. Proteins (50 μg) were then resolved by electrophoresis on 10% polyacrylamide gels containing SDS and electroblotted onto Protran BA83 cellulose nitrate membranes (Schleicher and Schuell, Dassel, Germany). The blots were blocked for 1 h at room temperature in TBS buffer (10 mM Tris pH 8.0, 150 mM NaCl and 0.5% Tween 20) containing 1% skim milk. The blots were then reacted with the rat anti-ABCG2 monoclonal antibody BXP-53 (at a dilution of 1:1,000; generously provided by Prof. R.J. Scheper and Dr G.L. Scheffer, VU University Medical Center, Amsterdam, The Netherlands). Blots were then rinsed three times in the same buffer each for 10 min at room temperature and reacted with second antibodies consisting either of horseradish peroxidase-conjugated goat anti-rat IgG (1:7,500 dilution, Jackson Immunoresearch Labs, West Grove, PA) for 1 h at room temperature. To normalize for loading differences, the nylon membranes were first stripped using the following procedure: the nylon membranes were incubated for 10 min in a stripping solution containing 0.5 M NaCl, 0.5 M acetic acid at pH 2.4. Then, the nylon membranes were washed twice with TBS and reacted overnight at 4°C with an affinity-purified rabbit polyclonal antiserum (anti-KETYY; at a 1:3,000 dilution; kindly provided by Prof. S.J. Karlish, Department of Biological Chemistry, The Weizmann Institute of Science, Rehovoth, Israel). Then, the blots were reacted with a second goat anti-rabbit IgG (1:15,000 dilution). Following three washes (each for 10 min) in TBS at room temperature, enhanced chemiluminescence detection was performed according to the manufacturer's instructions (Biological Industries, Beth-Haemek, Israel).

Flow cytometric assay of cellular tetramethylrosamine accumulation One-milliliter aliquots of cell suspension (5×10^5 cells/ml) in growth medium containing 25 mM Hepes at pH 7.3 were distributed into 1.5 ml polypropylene Eppendorf test tubes. Then, tetramethylrosamine was added to the growth medium at various concentrations of 10–1,000 nM as previously described [15]. To some test tubes which contained 1 μM tetramethylrosamine, 400 nM of the potent and specific ABCG2 efflux inhibitor Ko143 were added. After 30 min of incubation at 37°C, the test tubes were transferred to ice-cold water and centrifuged at 4°C. Cells were then suspended in ice-cold PBS containing 1% fetal calf serum and cellular fluorescence was determined using a flow cytometer. Excitation of tetramethylrosamine was at 550 nm and emission was collected at 574 nm.

Antifolate growth inhibition assay Cells (4×10^3–8×10^3 per well) were seeded in poly-D-lysine-coated 24-well plates in growth medium and then allowed to adhere to the substrate for 24 h at 37°C. Attached cells were then exposed to various concentrations of lipophilic antifolates and other cytotoxic agents for 72 h at 37°C, following which the drug-containing medium was aspirated. Finally, cells were detached by trypsinization and the number of viable cells was determined by a haemocytometer count after trypan blue staining.

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.

Results

ABCG2 expression in parental HEK293 cells and their R482-, G482- and T482 ABCG2 transfectants Western blot analysis was first performed in order to determine ABCG2 protein levels in membrane-rich preparations from parental HEK293 cells as well as their WT R482—and mutant G482—and T482 ABCG2 transfectants. Both the WT R482—as well as the mutant G482—and T482 ABCG2 transfectants displayed equal levels of ABCG2 overexpression, whereas ABCG2 was barely detectable in HEK293 cells (Fig. 2a). Reprobing the Western blot with an antibody to the abundant ABC transporter Na^+–K^+-ATPase (α subunit) confirmed that equal amounts of membrane proteins were being analyzed in the various cell lines (Fig. 2b).

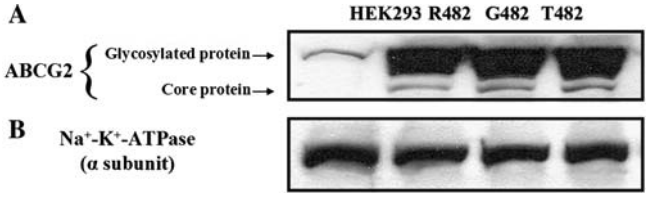

Fig. 2 Western blot analysis of ABCG2 levels in parental HEK293 cells and their WT R482—as well as mutant G482—and T482 ABCG2 transfectants. Triton X-100-soluble membrane proteins (50 μg) were resolved by electrophoresis on polyacrylamide gels containing SDS, electroblotted onto Protran BA83 cellulose nitrate membranes and reacted with the ABCG2-specific monoclonal antibody BXP-53 (**a**). Blots were then reacted with a second horseradish peroxidase-conjugated antibody and these nylon membranes were developed using a standard enhanced chemiluminescence procedure. To confirm that equal amounts of membrane proteins were being analyzed blots were stripped and reacted with an antibody to the α subunit of Na^+–K^+-ATPase (**b**)

Exclusion of tetramethylrosamine by mutant G482- and T482 ABCG2 transfectant cells but not by WT R482 ABCG2 Based upon the equal ABCG2 overexpression in the various transfectants, the ability of the WT R482 as well as the mutant G482- and T482 ABCG2 transfectants to extrude tetramethylrosamine was explored [15]. Remarkably, mutant G482- and T482 ABCG2 overexpressing cells were capable of a complete exclusion of tetramethylrosamine up to a 0.1 μM concentration (Fig. 3a). Moreover, tetramethylrosamine accumulation (at 0.1 μM) was restored to parental cell levels in both the G482- and T482 ABCG2 transfectants in the presence of 400 nM Ko143 (Fig. 3b), a specific and potent ABCG2 transport inhibitor [16]. These results establish the efficient tetramethylrosamine efflux capabilities of both the overexpressed G482- and T482 ABCG2 as opposed to WT R482 ABCG2.

Doxorubicin and paclitaxel resistance in the R482-, G482- and T482 ABCG2 transfectants Recent studies have shown that cells overexpressing the mutant G482 ABCG2 display high-level resistance to doxorubicin and can thereby be distinguished from their WT R482 counterpart that exhibits a low doxorubicin resistance [15, 25]. Consistently, the mutant T482- and G482 ABCG2 transfectants were 72- and 83-fold more resistant to doxorubicin than parental HEK293 cells, respectively, whereas the WT R482 ABCG2 transfectant displayed only 2.5-fold resistance (Table 1); doxorubicin resistance was reversed in all these ABCG2 transfectants by Ko143 (Table 1). Interestingly however, both the WT R482 ABCG2 as well as the mutant G482- and T482 ABCG2 overexpressing cells displayed a similar level of resistance (24-, 31- and 33-fold, respectively) to the microtubule stabilizing agent, paclitaxel, relative to parental HEK293 cells (Table 1). Hence whereas the mutant ABCG2 transporters

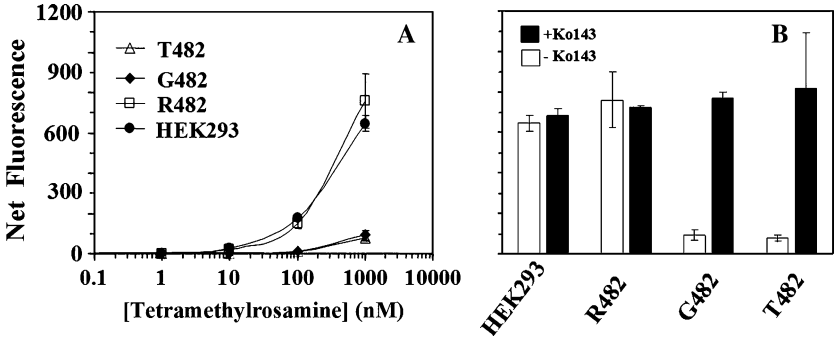

Fig. 3 Accumulation of tetramethylrosamine in HEK293 cells and their R482-, G482- and T482 ABCG2 transfectants in the absence or presence of Ko143. Cells were first detached by trypsinization, suspended in a 20 mM HEPES (pH 7.4)-buffered medium containing various tetramethylrosamine concentrations ranging from 1 nM to 1 μM and incubated for 30 min at 37°C (**a**). Another portion of cells was incubated with 100 nM tetramethylrosamine in absence or presence of 400 nM Ko143 for 30 min at 37°C (**b**). Cells were then washed once with ice-cold medium and analyzed by flow cytometry for mean fluorescence per cell. Results depicted represent net mean fluorescence values (i.e., after subtraction of autofluorescence of unstained cells) obtained from three different experiments

Table 1 Summary of growth inhibition studies upon 72 h exposure to various anticancer drugs in the absence or presence of Ko143

Drug (target enzyme)	Cell line (IC_{50} (nM))							
	HEK293		R482		G482		T482	
	−Ko143	+Ko143	−Ko143	+Ko143	−Ko143	+Ko143	−Ko143	+Ko143
AG337 (TS)	1,400±100 (1.0)	1,300±600 (1.0)	5,300±1,700 (3.8)	2,900±1,100 (2.2)	147,600±12,200 (105.4)	2,250±500 (1.7)	148,700±3,600 (106.2)	2,725±600 (2.1)
AG331 (TS)	1.8±0.6 (1.0)	2.1±0.9 (1.0)	3.0±1 (1.7)	3.6±1.7 (1.7)	9.5±2.3 (5.3)	2.1±0.9 (1.0)	8±0.9 (4.4)	3.8±0.4 (1.8)
AG377 (TS/DHFR)	1.7±0.6 (1.0)	1.3±0.6 (1.0)	10±3.6 (5.9)	2±0.1 (1.5)	64±10.4 (37.6)	1.7±0.5 (1.3)	93.7±12.1 (55.1)	3.4±0.8 (2.6)
Trimetrexate (DHFR)	42.9±4.5 (1.0)	41.8±3.6 (1.0)	271±32 (6.3)	64.9±18.2 (1.6)	1,508±19 (35.2)	70.3±12.7 (1.7)	2,727±801 (63.6)	89.1±42.8 (2.1)
Piritrexim (DHFR)	67±10.1 (1.0)	59±12.5 (1.0)	105±23.6 (1.6)	75.6±4.7 (1.3)	822±84.5 (12.3)	69±17 (1.2)	749±12.8 (11.2)	98±2.8 (1.7)
Pyrimethamine (DHFR)	2,320±200 (1.0)	1,960±80 (1.0)	15,700±400 (6.8)	1,940±160 (1.0)	5,500±520 (2.4)	6,100±220 (3.1)	4,385±1,022 (1.9)	2,773±448 (1.4)
Metoprine (DHFR)	800±13.5 (1.0)	642±37.8 (1.0)	745±167 (0.9)	809±114 (1.3)	656±44.3 (0.8)	318±24.8 (0.5)	1,047±260 (1.3)	962±193 (1.5)
Doxorubicin	4.8±1.2 (1.0)	4.1±1.2 (1.0)	12.1±2.4 (2.5)	3.9±1.1 (1.0)	396±117 (82.5)	14.8±4.2 (3.6)	347±99 (72.3)	7.2±2.8 (1.8)
Paclitaxel	0.74±0.05 (1.0)	0.69±0.08 (1.0)	18±1.9 (24.3)	0.87±0.19 (1.3)	22.8±3 (30.8)	5±1.3 (7.2)	24.6±5.9 (33.2)	2.4±0.5 (3.5)
Cisplatinum	431±185 (1.0)	450±212 (1.0)	290±55 (0.7)	431±284 (1.0)	417±205 (1.0)	405±215 (0.9)	451±270 (1.0)	633±377 (1.4)
5-Fluorouracil	2,059±166 (1.0)	2,266±324 (1.0)	2,827±247 (1.4)	2,897±84.1 (1.3)	2,644±321 (1.3)	2,710±281 (1.2)	2,388±289 (1.4)	2,869±301 (1.3)

Fold resistance is given in parenthesis

conferred a preferential resistance to doxorubicin, paclitaxel resistance was not substantially affected by the presence of an R-, G- or T at amino acid 482.

Lipophilic antifolate resistance in transfectants overexpressing the mutant G482- and T482 ABCG2 We have recently shown that upon a 4 h drug exposure, cells overexpressing the mutant G482 ABCG2 displayed 120–6,250-fold resistance to various hydrophilic antifolates that rely on the RFC for their cellular entry [15]. In contrast, upon a continuous (72 h) drug exposure, both the WT R482- and G482 ABCG2 cells lost almost all their resistance to hydrophilic antifolates. Therefore, we here examined the ability of the WT R482 as well as the mutant G482- and T482 ABCG2 to confer resistance to a continuous (72 h) exposure to various lipophilic antifolates (Fig. 1) that have been shown to rapidly enter cells by diffusion in an RFC-independent manner [17, 18, 23, 26]. Mutant G482 ABCG2 overexpressing cells displayed 105-, 38-, 35-, 12- and 5-fold resistance to the lipophilic antifolates AG337 (Thymitaq; Nolatrexed), AG377, Trimetrexate (Neutrexin), Piritrexim and AG331, respectively, relative to parental HEK293 cells (Fig. 4a–e). Likewise, cells overexpressing the mutant T482 ABCG2 exhibited 106-, 64-, 55-, 11- and 4-fold resistance to AG337, Trimetrexate, AG377, Piritrexim and AG331, respectively (Table 1). Moreover, this lipophilic antifolate resistance in G482- (Fig. 4f–j) and T482 ABCG2 overexpressing cells was reversed by Ko143 (Table 1). In contrast, cells overexpressing the WT R482 ABCG2 showed a low-level resistance (1.6–6.3-fold) to these lipophilic antifolates; this low-level resistance was fully reversed by the ABCG2 drug efflux inhibitor Ko143 (Table 1). Remarkably, the mutant G482- and T482 ABCG2 transfectants either retained parental cell sensitivity or displayed only low-level resistance to the low molecular weight 2,4-diaminopyrimidine lipophilic antifolates metoprine and pyrimethamine (Fig. 5a, b) regardless of the presence of Ko143 (Fig. 5c, d and Table 1). In order to provide further support to the lack of lipophilic antifolate resistance in cells overexpressing the WT (R482) ABCG2, we used MCF-7/MR cells that were established by stepwise selection to mitoxantrone [27]. Whereas these MCF-7/MR cells with R482 overexpression [28] displayed ~60-fold resistance to the hydrophilic antifolate MTX as well as 22–43-fold resistance to *Vinca* alkaloids and mitoxantrone, they retained parental sensitivity to the lipophilic antifolates AG377 and trimetrexate (Table 2). These results provide the first evidence that the mutant G482- and T482 ABCG2 but not the WT R482 ABCG2 confer high-level resistance to certain lipophilic antifolates upon a continuous drug exposure.

Parental sensitivity of G482-, T482 and R482 ABCG2 to cisplatinum and 5-fluorouracil The current growth inhibition study with lipophilic antifolates was extended to established non-antifolate hydrophilic cytotoxic drugs including the alkylating agent cisplatinum and the fluoropyrimide 5-fluorouracil. Both the WT R482 and

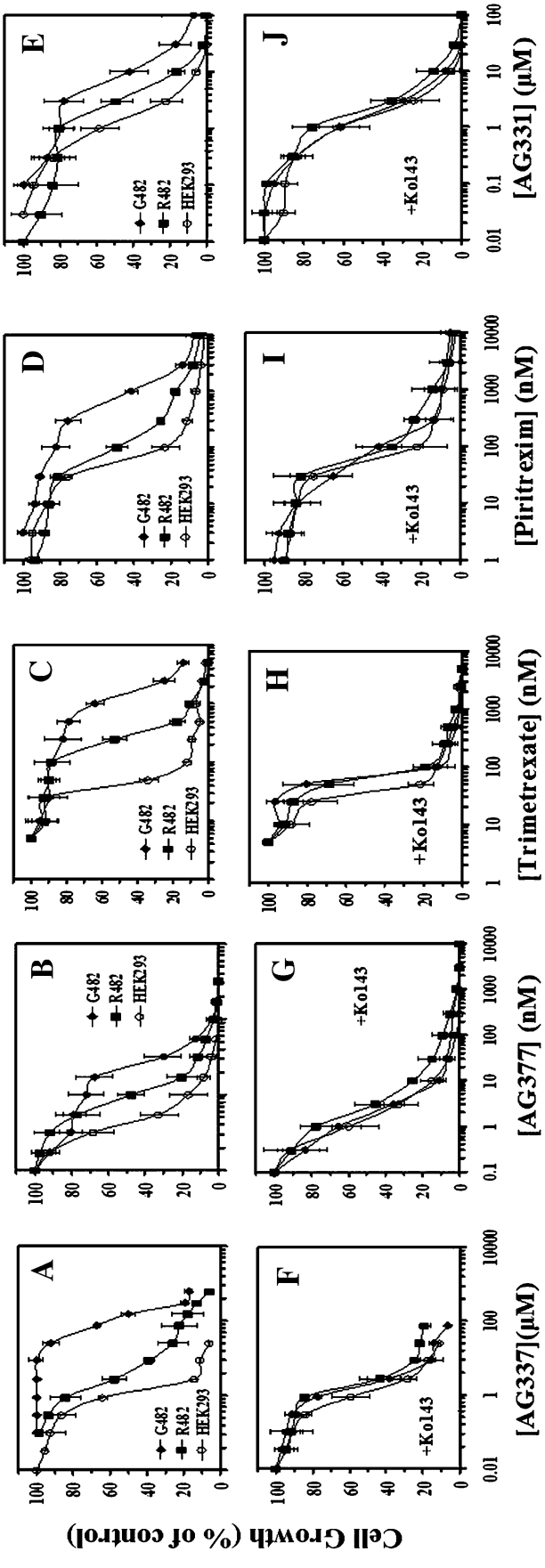

Fig. 4 Cytotoxicity of AG337, AG377, AG331, Trimetrexate and Piritrexim to HEK293 cells and their R482-, G482- and T482 ABCG2 transfectants in the presence or absence of Ko143. One portion of parental HEK293 cells, their G482- and R482 ABCG2 transfectants was exposed for 72 h to various concentrations of AG337 (**a**), AG377 (**b**), Trimetrexate (**c**), Piritrexim (**d**) and AG331 (**e**) in the absence of Ko143. Whereas, another portion of cells was continuously incubated with these lipophilic antifolates in the presence of 400 nM Ko143 (**f**, **g**, **h**, **i** and **j**, respectively). The number of viable cells was then determined using trypan blue exclusion. Results depicted are means ± SD obtained from three to six independent experiments

mutant G482- and T482 ABCG2 transfectants displayed parental cell (HEK293) sensitivity to these cytotoxic agents regardless of whether Ko143 was present (Table 1). These results suggest that cells overexpressing the R482-, G482- and T482 ABCG2 can be readily eradicated by certain cytotoxic drugs including cisplatinum and 5-fluorouracil that are neither recognized by the mutant G482- and T482 ABCG2 nor by the WT R482 ABCG2.

Discussion

Our current findings support the conclusion that the mutant G482- and T482 ABCG2 are important determinants of resistance to a continuous exposure to various lipophilic antifolates. This conclusion is supported by two main findings; first, transfectant cells overexpressing the mutant G482- and T482 ABCG2 conferred up to two orders of magnitude of resistance to various lipid-soluble antifolates most notably AG337 (Thymitaq; Nolatrexed), AG377 and Trimetrexate (Neutrexin). In contrast, despite the similar levels of transporter overexpression, the WT R482 ABCG2 conferred only a low-level resistance to lipophilic antifolates. Second, the high-level resistance to lipophilic antifolates displayed by cells overexpressing the mutant G482- and T482 ABCG2 was reversed by Ko143, a potent and specific ABCG2 inhibitor [16]. Consistently, the modest resistance levels conferred by the WT R482 ABCG2 were also reversible by Ko143. These results suggest that the mutant G482- and T482 ABCG2 mediate a Ko143-sensitive efflux of various lipophilic antifolates thereby resulting in drug resistance. This conclusion is consistent with our current findings [15] that G482 ABCG2 conferred high-level resistance to various hydrophilic antifolates and that this drug resistance was fully reversed by the specific and potent ABCG2 drug efflux inhibitor Ko143.

The lipid-soluble antifolates studied here possess several characteristics, which potentially have important implications to the mechanism of anticancer drug efflux mediated by the mutant G482- and T482 ABCG2 transporters. First, the lipophilic antifolates studied here have a calculated log *P* (i.e., Log of octanol: water partition

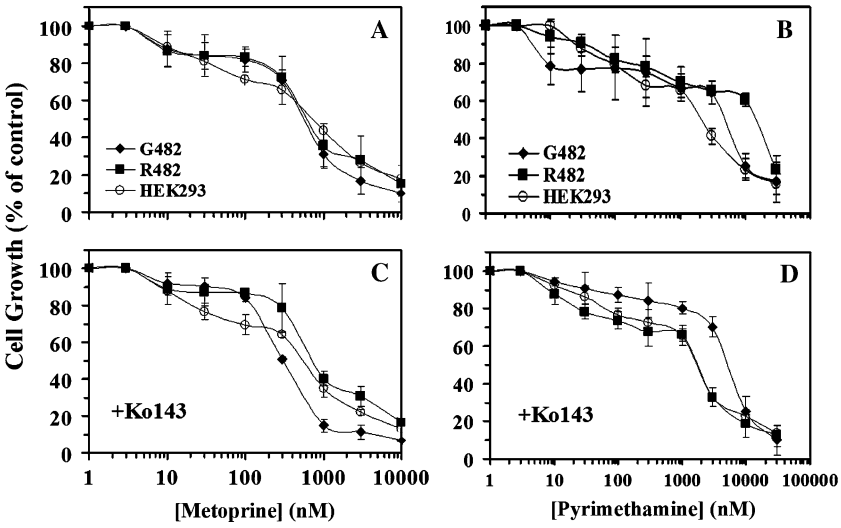

Fig. 5 Cytotoxicity of metoprine and pyrimethamine to HEK293 cells and their R482 and G482 ABCG2 transfectants. Parental HEK293 cells and their R482- and G482 ABCG2 transfectant cells were exposed for 72 h to various concentrations of metoprine (**a**) or pyrimethamine (**b**) in the absence of Ko143. Whereas, another portion of cells was incubated continuously with these lipophilic antifolates in the presence of 400 nM Ko143 (**c** and **d**, respectively). Cells were then washed and viable cell numbers were determined. Results depicted are means ± SD obtained from three to five independent experiments

Table 2 Summary of growth inhibition studies upon 4 or 72 h exposure to various anticancer drugs in the absence or presence of Ko143

Drug (target enzyme)	Cell line (IC_{50} (nM))				
	MCF-7		MCF-7/MR		Reference
	−Ko143	+Ko143	−Ko143	+Ko143	
Methotrexate[a] (DHFR)	3,300±1,300 (1.0)		196,000±9,200 (59.4)		Ifergan et al. [28]
AG377 (TS/DHFR)	5.7±1.13 (1.0)	6.7±1.3 (1.0)	5.8±1.4 (1.0)	5.7±1.2 (0.85)	Current paper
Trimetrexate (DHFR)	61.5±13.2 (1.0)	40±15.6 (1.0)	62.3±3.8 (1.0)	67.5±26.2 (1.1)	Current paper
Mitoxantrone	17.6±0.4 (1.0)	8.2±0.5 (1.0)	602±89.8 (34.2)	36.7±1.0 (4.5)	Ifergan et al. [28]
Doxorubicin	400 (1.0)		3,300 (8.3)		Taylor et al. [27]
Vincristine	3.9 (1.0)		86 (22.0)		Taylor et al. [27]
Vinblastine	7.0 (1.0)		300 (43.0)		Taylor et al. [27]

Fold resistance is given in parenthesis
[a] 4 h drug exposure

coefficient) of 2.24–3.53 and an experimental log *P* in the range of 1.85–2.69 [29]. This implies a ~100–1,000-fold preferential solubility in the lipid phase of biomembranes (e.g., the plasma membrane) than in water [17, 18, 23, 24]. This is in a sharp contrast to the hydrophilic antifolate MTX with an experimental log *P* value of −1.85 thereby resulting in a ~100-fold better water-solubility than lipid-solubility [29]. Second, lipophilic antifolates rapidly enter cells by passive diffusion and are completely independent of the RFC for their cellular entry [17, 18, 23, 24]. In support of this is the finding that multiple hydrophilic antifolate-resistant cell lines with loss of RFC transport function are highly hypersensitive to lipid-soluble antifolates including trimetrexate and AG377 [21, 22]. Third, unlike polyglutamatable hydrophilic antifolates, lipophilic antifolates lack a glutamate residue and thus cannot undergo polyglutamylation and are in fact bound to their intracellular target enzyme(s) as well as to biomembranes and certain hydrophobic proteins. Hence, once the drug is at the extracellular milieu, all the features mentioned above favor the 100–1,000 preferential co-partition of these lipophilic antifolates in the lipid phase of the plasma membrane. Based on the present finding that G482-, T482 and R482 ABCG2 were significantly overexpressed at the plasma membrane [15], it is possible that the G482- and T482 ABCG2 transporters recognize lipophilic antifolates within the lipid bilayer and then extrude them out of cells even before they could traverse the plasma membrane. According to this intriguing possibility, G482- and T482 ABCG2 resemble Pgp (ABCB1), which has been shown to function as an MDR efflux transporter extruding multiple structurally dissimilar hydrophobic cytotoxic drugs from within the lipid bilayer [30] including Trimetrexate and Piritrexim [20, 31]. Hence, according to this scenario and like Pgp, G482- and T482 ABCG2 must be efficient extrusion pumps with a lipophilic antifolate efflux rate exceeding the passive diffusion rate of lipophilic antifolates. This presumption may be consistent with the high transport Vmax of 2.4 nmol/min/mg protein measured for MTX transport in isolated vesicles with R482 ABCG2 overexpression [32]. Furthermore, previous transport studies with radiolabeled MTX (and folic acid) in membrane vesicles purified from ABCG2 overexpressing cells revealed a transport Km in the millimolar range (i.e., 0.68–1.34 mM) [32, 33]. Although this concentration may seem to be clinically high (except for example with the high dose MTX treatment in osteosarcoma and acute lymphoblastic leukemia), a lipophilic

antifolate with an average log *P* value of 2 and at an extracellular concentration of 10–100 μM will rapidly achieve an effective concentration of 1–10 mM in the lipid bilayer of the plasma membrane. Hence, the G482- and T482 ABCG2 overexpressed in the plasma membrane may have the opportunity of interacting with lipophilic antifolates at the Km value or even at 10-fold above the Km. It is possible that the two orders of magnitude increase in the membrane concentration of the lipophilic antifolate relative to its extracellular concentration may be a contributing factor to the marked lipophilic antifolate resistance found in cells overexpressing the G482- and T482 ABCG2.

The present growth inhibition studies revealed that overexpression of the mutant G482- and T482 ABCG2 transporters conferred resistance to AG337, AG377, Trimetrexate and Piritrexim (Fig. 4a–e and Table 1). In contrast, both the WT and mutant transporters failed to confer any major resistance to the 2,4-diaminopyrimidines metoprine and pyrimethamine (Fig. 5a, b). Furthermore, recently we have shown that overexpression of the mutant G482 ABCG2 conferred high-level resistance to the hydrophilic folic acid analogues MTX, Tomudex and GW1843 [15]. Hence, it appears that the mutant G482- and T482 ABCG2 presumably have the ability to extrude the three rings (or more) structure of folic acid, hydrophilic antifolates like MTX, Tomudex, GW1843 as well as the lipophilic antifolates AG337, AG377, Trimetrexate and Piritrexim (Fig. 1). In contrast, low molecular weight lipophilic antifolates including metoprine and pyrimethamine of the 2,4-diaminopyrimidine family with only 2 aromatic rings (Fig. 1) and thus much less bulky structure were neither recognized by G482-, T482 nor by the WT R482 ABCG2. Thus, it is possible that the putative ABCG2 pharmacophore may recognize a certain minimal substrate size and/or overall molecular bulk that does not appear to be below a three rings member structure. In fact, this suggestion is consistent with a recent paper which explored the ability to predict Pgp substrates by examining molecular bulk as quantified by two objective parameters: calculated molar refraction (CMR) and molecular weight [34]. In the latter study it was found that the various hydrophobic Pgp substrates had an optimal CMR value of 6–15, an optimal molecular weight of 350–550 as well as an optimal log *P* value of 3–5. Consistently, it is interesting to note that, to date, none of the published transport substrates of G482–T482 and WT R482 ABCG2 had less than a three aromatic rings structure [1, 10, 11]. This finding may have important clinical implications for the overcoming of anticancer drug resistance in tumors that may harbor G482- and T482 ABCG2 overexpression. Thus, we find here that transfectant cells overexpressing the WT R482- and mutant G482- and T482 ABCG2 retained parental cell sensitivity not only to metoprine and pyrimethamine (Fig. 5a, b) but also to well-established anticancer drugs like cisplatinum and 5-fluorouracil (Table 1). These results strongly suggest that MDR tumors harboring the WT R482 or mutant G482- and T482 ABCG2 overexpression may be readily eradicated by such low molecular weight cytotoxic agents that are recognized by none of these MDR efflux transporters.

In our previous report [15] as well as in the current study we find a tight correlation between the ability of the mutant G482- and T482 ABCG2 to efficiently extrude the lipophilic chromophore tetramethylrosamine and their capacity to confer resistance to hydrophilic and lipophilic antifolates. Specifically, transfectant cells overexpressing both the mutant G482- and T482 ABCG2 extruded tetramethylrosamine up to an extracellular concentration of 0.1 μM whereas the WT R482 ABCG2 failed to do so and therefore accumulated this chromophore in a dose-dependent manner. The functional role of the mutant ABCG2 in mediating this chromophore exclusion was verified using the potent and specific ABCG2 efflux inhibitor Ko143. Hence these results suggest that this 30 min tetramethylrosamine accumulation flow cytometric assay in the absence or presence of Ko143 may be clinically used for the identification of tumor-derived cells overexpressing these mutant ABCG2 transporters. This may facilitate the identification of clonal tumor-derived cell populations harboring a dominant MDR phenotype to various cytotoxic agents including hydrophilic and lipophilic antifolates. As such, the outcome of this study may contribute to further improve the efficacy of lipophilic antifolates as cancer chemotherapeutic drugs. Another possible implication of this tetramethylrosamine extrusion assay relates to the putative ability of the mutant G482- and T482 ABCG2 but not the WT R482 pharmacophore to bind this chromophore within the lipid phase of the plasma membrane, where it reaches high concentrations as a hydrophobic compound [35]. We here suggest that the putative structural alteration occurring in the G482- and T482 ABCG2 due to the elimination of a basic amino acid (i.e., arginine) and/or the insertion of a different amino acid presumably facilitates the formation of an ABCG2 pharmacophore that opens to the lipid phase of the plasma membrane thereby allowing for an efficient hydrophobic chromophore binding and extrusion. In contrast, the WT R482 ABCG2 transporter presumably lacks this altered pharmacophore structure and therefore may fail to bind and/or extrude this hydrophobic chromophore. This putative mechanistic scenario may possibly hold true for lipophilic antifolate binding by the G482- and T482 pharmacophore but not by the R482 ABCG2.

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References

1. Borst P, Elferink RO (2002) Mammalian ABC transporters in health and disease. Annu Rev Biochem 71:537–592
2. Dean M, Harmon Y, Chimini G (2001) The human ATP-binding cassette (ABC) transporter superfamily. J Lipid Res 42:1007–1017
3. Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE, Gottesman MM (2003) P-glycoprotein: from genomics to mechanism. Oncogene 22:7468–7485
4. Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258:1650–1654
5. Kruh GD, Belinsky MG (2003) The MRP family of drug efflux pumps. Oncogene 22:7535–7552
6. Haimeur A, Conseil G, Deeley RG, Cole SP (2004) The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation. Curr Drug Metab 5:21–53
7. Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, Ross DD (1998) A multidrug resistance transporter from MCF-7 breast cancer cells. Proc Natl Acad Sci USA 95:15665–15670
8. Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, Dean M (1998) A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res 58:5337–5339
9. Miyake K, Mickley L, Litman T, Zhan Z, Robey RW, Christensen B, Brangi M, Greenberger L, Dean M, Fojo T, Bates SE (1999) Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. Cancer Res 59:8–13
10. Sarkadi B, Ozvegy-Laczka O, Nemet K, Varadi A (2004) ABCG2—a transporter for all seasons. FEBS Lett 567:116–120
11. Staud F, Pavek P (2005) Breast cancer resistance protein (BCRP/ABCG2). Int J Biochem Cell Biol 37:720–725
12. Kage K, Tsukahara S, Sugiyama T, Asada S, Ishikawa E, Tsuruo T, Sugimoto Y (2002) Dominant-negative inhibition of breast cancer resistance protein as a drug efflux pump through the inhibition of S–S dependent dimerization. Int J Cancer 97:626–630
13. Xu J, Liu Y, Yang Y, Bates SE, Zhang JT (2004) Characterization of oligomeric human half-ABCC transporter ATP-binding cassette G2. J Biol Chem 279:19781–19789
14. Polgar O, Robey RW, Morisaki K, Dean M, Michejda C, Sauna ZE, Ambudkar SV, Tarasova N, Bates SE (2004) Mutational analysis of ABCG2: role of the GXXXG motif. Biochemistry 43:9448–9456
15. Shafran A, Ifergan I, Bram E, Jansen G, Kathmann I, Peters GJ, Robey RW, Bates SE, Assaraf YG (2005) ABCG2 harboring the Gly482 mutation confers high level resistance to hydrophilic antifolates. Cancer Res 65:8414–8422
16. Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, Schellens JH, Kooman GJ, Schinkel AH (2002) Potent and specific inhibition of breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. Mol Cancer Ther 1:417–425
17. Duch DS, Edelstein MP, Bowers SW, Nichol CA (1982) Biochemical and chemotherapeutic studies on 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-d]pyrimidine (BW 301U), a novel lipid-soluble inhibitor of dihydrofolate reductase. Cancer Res 42:3987–3994
18. Fry DW, Besserer JA (1988) Characterization of trimetrexate transport in human lymphoblastoid cells and development of impaired influx as a mechanism of resistance to lipophilic antifolates. Cancer Res 48:6986–6991
19. Bertino JR (1993) Ode to methotrexate. J Clin Oncol 11:5–14
20. Assaraf YG, Molina A, Schimke RT (1989) Cross-resistance to the lipid-soluble antifolate trimetrexate in human carcinoma cells with the multidrug resistance phenotype. J Natl Cancer Inst 81:290–294
21. Rothem L, Ifergan I, Kaufman Y, Priest DG, Jansen G, Assaraf YG (2002) Resistance to multiple novel antifolates is mediated via defective drug transport resulting from clustered mutations in the reduced folate carrier gene in human leukemia cells. Biochem J 367:741–750
22. Liani E, Rothem L, Bunni MA, Smith CA, Jansen G, Assaraf YG (2003) Loss of folylpoly-γ-glutamate synthetase activity is a dominant mechanism of resistance to polyglutamylation-dependent novel antifolates in multiple human leukemia sublines. Int J Cancer 103:587–599
23. Purcell WT, Ettinger DS (2003) Novel antifolate drugs. Curr Oncol Rep 5:114–125
24. Chu E, Callender MA, Farrell MP, Schmitz JC (2003) Thymidylate synthase inhibitors as anticancer agents: from bench to bedside. Cancer Chemother Pharmacol 52:S80–S89
25. Robey RW, Honjo Y, Morisaki K, Nadjem TA, Runge S, Risbood M, Poruchynsky MS, Bates SE (2003) Mutations at amino-acid 482 in the ABCG2 gene affect substrate and antagonist specificity. Br J Cancer 89:1971–1978
26. Westerhof GR, Schornagel JH, Kathmann I, Jackman AL, Rosowsky A, Forsch RA, Hynes JB, Boyle FT, Peters GJ, Pinedo HM, Jansen G (1995) Carrier- and receptor-mediated transport of folate antagonists targeting folate-dependent enzymes: correlates of molecular-structure and biological activity. Mol Pharmacol 48:459–471
27. Taylor CW, Dalton WS, Parrish PR et al (1991) Different mechanisms of decreased drug accumulation in doxorubicin and mitoxantrone resistant variants of the MCF-7 human breast cancer cell line. Br J Cancer 63:923–929
28. Ifergan I, Shafran A, Jansen G, Hooijberg JH, Scheffer GL, Assaraf YG (2004) Folate deprivation results in the loss of breast cancer resistance protein (ABCG2) expression: a role for BCRP in cellular folate homeostasis. J Biol Chem 279:25527–25534
29. Hansch C, Hoekman D, Leo A, Zhang L, Li P (1995) The expanding role of quantitative structure–activity relationships (QSAR) in toxicology. Toxicol Lett 79:45–53
30. Borgnia MJ, Eytan GD, Assaraf YG (1996) Competition of hydrophobic peptides, cytotoxic drugs and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its ATPase activity. J Biol Chem 271:3163–3171
31. Assaraf YG, Molina A, Schimke RT (1989) Sequential amplification of dihydrofolate reductase and multidrug resistance genes in Chinese hamster ovary cells selected for stepwise resistance to the lipid-soluble antifolate trimetrexate. J Biol Chem 264:18326–18334
32. Volk EL, Schneider E (2003) Wild-type breast cancer resistance protein (BCRP/ABCG2) is a methotrexate polyglutamate transporter. Cancer Res 63:5538–5543
33. Chen Z-S, Robey RW, Belinsky MG, Schvaveleva I, Ren XO, Sugimoto Y, Ross DD, Bates SE, Kruh GD (2003) Transport of methotrexate, methotrexate polyglutamates and 17beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. Cancer Res 63:4048–4054
34. Gombar VK, Polli JW, Humphreys JW, Wring AW, Serabjit-Singh CS (2004) Predicting P-glycoprotein substrates by a quantitative structure–activity relationship model. J Pharm Sci 93:957–968
35. Eytan GD, Regev R, Oren G, Hurwitz C, Assaraf YG (1997) Efficiency of P-glycoprotein-mediated exclusion of rhodamine dyes from multidrug resistant cells is determined by their passive transmembrane movement rate. Eur J Biochem 248:104–112