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# Research Article

# Purification of breast cancer resistance protein ABCG2 and role of arginine-482

**A. Pozza [a, †], J. M. Perez-Victoria [b, †], A. Sardo [a], A. Ahmed-Belkacem [a] and A. Di Pietro [a, *]**

[a] Institut de Biologie et Chimie des Protéines, UMR5086 CNRS-Université de Lyon et IFR128 BioSciences Lyon-Gerland, 7 Passage du Vercors, 69367 Lyon Cedex 07 (France), Fax: +33 4 7272 2604, e-mail: a.dipietro@ibcp.fr
[b] Instituto de Parasitologia y Biomedicina 'Lopez-Neyra', Consejo Superior de Investigaciones Cientificas, Granada (Spain)

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**Abstract.** Human ABCG2 was efficiently overexpressed in insect cell membranes, solubilized with 3-[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonate, and purified through *N*-terminal hexahistidine tag. Its functionality was assessed by high vanadate-sensitive ATPase activity, and nucleotide-binding capacity. Interestingly, the R482T point mutation increased both maximal hydrolysis rate and affinity for MgATP, and lowered sensitivity to vanadate inhibition. Direct nucleotide binding, as monitored by quenching of intrinsic fluorescence, indicated a mutation-related preference for ATP over ADP. The R482T mutation only produced a limited change, if any, on the binding of drug substrates, indicating that methotrexate, on the one hand, and rhodamine 123 or doxorubicin, on the other hand, bound similarly to wild-type and mutant transporters whether or not they were subject to cellular transport. In addition, the characteristic inhibitors GF120918 and 6-prenylchrysin, which alter mitoxantrone efflux much better for wild-type than mutant ABCG2, bound similarly to purified ABCG2, while the highly-potent Ko143 bound in the nanomolar range also effective in inhibition of drug transport. All results indicate that the role of the arginine-482 mutation on substrate drug transport and inhibitor efficiency is not mediated by changes in drug binding.

**Keywords.** Breast cancer resistance protein, multidrug resistance, ABC transporter, substrates, inhibitors, ATPase activity, anticancer chemotherapy.

## Introduction

The multidrug resistance (MDR) phenotype of cancer cells has been often related to overexpression within plasma membranes of ATP-binding cassette (ABC)-transporters able to efflux many chemotherapeutic drugs at the expense of ATP hydrolysis. More recently, in addition to P-glycoprotein/ABCB1 [1] and MRP1 (multidrug resistance protein)/ABCC1 [2], breast cancer resistance protein/ABCG2 has been shown to be involved [3–5]. Despite being a half-transporter, functioning at least as a homodimer, ABCG2 exhibits a pattern of transported drugs largely overlapping with P-glycoprotein (for a review see [6–8]). In contrast, only a few inhibitors, such as GF120918, are efficient on both transporters [9], whereas a few compounds, namely fumitremorgin C [10] and its potent derivative Ko143 [11], 6-prenylchrysin [12] and novobiocin [13], appear to be ABCG2 specific (for a recent review see [14]).

Only limited information is available on the transporter molecular mechanism, partially due to the lack of purified protein and three-dimensional structure, despite some tentative extrapolations from extensive functional

* Corresponding author.
† These authors contributed equally to this work.

data with P-glycoprotein and MRP1, and high-resolution structure of MsbA, a bacterial lipid exporter [15]. Interestingly, a hot-spot mutation substituting arginine-482, especially by threonine or glycine, was shown to markedly change the spectrum for substrate drugs, by abolishing methotrexate transport [16, 17] and, on the contrary, allowing transport of both rhodamine 123 and doxorubicin [18–21], or increasing that of mitoxantrone [20, 21]. Transport of topotecan, SN-38, Hoechst33342 and pheophorbide a appeared much less affected [18, 20–22] (for a review, see [8]). A number of other mutations at the same position have shown that the positive charge of arginine is not alone a decisive parameter [19, 23, 24]. Interestingly, the mutation was also found to alter the efficiency of some inhibitors, such as novobiocin against BODIPY-prazosin and topotecan transport [21], or GF120918 and 6-prenylchrysin against mitoxantrone transport [12]. In addition, the ATPase activity of insect cell membranes enriched with recombinant human ABCG2 appeared to be dependent on mutation at position 482 [12, 20, 25].

Since arginine-482 is assumed to be located at the inner edge of the third membrane span of ABCG2 transmembrane domain, it might occupy a strategic position towards either binding or transport of substrate drugs, or energy coupling. To decide between these alternatives and establish structure-function relationships, the aim of this study was to overexpress and purify both wild-type (R482) and mutant (R482T) ABCG2 to determine parameters of ATPase activity and measure direct binding of ligands, *i.e.* nucleotides, substrate drugs and inhibitors. Changes in intrinsic fluorescence have been used to monitor direct ligand interaction. Each ABCG2 monomer contains a total of five tryptophan residues, and the intrinsic fluorescence of various ABC transporters have been found to be quite sensitive to quenching upon interaction with many ligands in both recombinant nucleotide-binding domains (mouse P-glycoprotein [26–28], human MRP1 [29] or parasite Ltmdr1[30]) and full transporters (rodent P-glycoprotein [27, 31] or yeast Pdr5p [32]).

The present results show that the R482T mutation represents a gain-of-function for ATPase activity by increasing both maximal rate of hydrolysis and affinity for MgATP. The interaction with any nucleotide, substrate drug or inhibitor induces a quenching of intrinsic fluorescence, allowing the binding of the different ligands to be characterized. The mutation does not alter the binding of either substrate drugs or inhibitors, suggesting a role of arginine-482 in the transport of substrate drugs.

## Materials and methods

**Chemical compounds.** ATP, ADP, 1,4-dithio-DL-threitol, protease inhibitors cocktail, mitoxantrone, methotrexate, rhodamine 123 and novobiocin were purchased from Sigma. Sodium orthovanadate was from Aldrich. Doxorubicin, Hoechst33342 and azolectin were from Fluka. Pheophorbide a was purchased from Frontier Scientific (Logan, USA). 6-Prenylchrysin and GF120918 were obtained as previously described [12], and Ko143 was kindly provided by Dr. A.H. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands).

**cDNA cloning and site-directed mutagenesis.** The *pcDNA3* plasmid containing *R482T-ABCG2* cDNA, provided by Dr. D. D. Ross, was used for subcloning into the *pTriex-4-Neo* plasmid (Novagen, VWR, Fontenay-sous-Bois, France). The *R482T* cDNA was mutated to obtain *R482* cDNA by site-directed mutagenesis using a QuickChange Site-directed mutagenesis Kit (Stratagene, La Jolla). The primers used were: 5′-CTGTTATCTGATTTATTACCCATGAGGATGTTACCAAGTATTATATTTACC-3′ and 5′-GGTAAATATAATACTTGGTAACATCCTCATGGGTAATAAATCAGATAACAG-3′.

**Generation of recombinant baculoviruses.** Recombinant baculoviruses carrying either *R482T-* or *R482-ABCG2* human cDNA were generated with a BacVector transfection kit (Novagen), according to manufacturer's instructions. Individual clones expressing the protein were isolated by plaque staining with neutral red, and the clone producing the recombinant protein at the highest yield was selected by Western blot (see below). The selected recombinant baculoviruses were amplified with Sf9 insect cells.

**Protein overproduction and inverted membrane vesicle preparation.** High Five insect cells were infected with recombinant baculoviruses at a multiplicity of 20 for 2 h. After 3 days, the cells were harvested, and their membranes isolated as described [33].

**SDS-PAGE and Western blot.** Protein concentration of inverted membrane vesicles was determined by the modified Lowry method with bovine serum albumin as a standard. Samples were not heated before loading, and protein contents were analyzed by SDS-PAGE in 8% or 10% polyacrylamide gels. The gels were either stained with Coomassie brilliant blue G-250, or used for protein electrotransfer (at 90 mA for 1 h) and Western blot onto 0.45- µm pore nitrocellulose filters (Bio-Rad). Proteins of interest were detected by the anti-ABCG2 antibody BXP-21 (1 : 1000 dilution, for 1 h at room temperature) in accordance with manufacturer's instructions (Alexis); the secondary antibody was a goat anti-mouse IgG (H + L)-alkaline phosphatase (AP) conjugate (Bio-Rad) used at 1 : 2300 dilution for 1 h at room temperature. Detection was performed using the AP conjugate substrate kit (Bio-Rad) according to manufacturer's instructions.

**Protein purification.** Inverted membrane vesicles of High Five cells infected by baculovirus vector encoding the R482 or R482T transporter were treated with solubilization buffer {50 mM HEPES/NaOH, pH 8, 18 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5 M NaCl, 10 mM imidazole, 20% glycerol} at a final protein concentration of 2 mg/ml, for 30 min with gently shaking at 4 °C. The sample was then centrifuged at 15 000 *g* for 30 min at 4 °C. The supernatant containing solubilized protein was immediately applied onto a Ni-NTA agarose (Qiagen) resin, which was pre-equilibrated in the solubilization buffer, for 2 h at 4 °C with gently shaking. The Ni-NTA agarose resin with bound protein was washed with ten column volumes of wash buffer (50 mM HEPES/NaOH, pH 8, 18 mM CHAPS, 0.5 M NaCl, 30 mM imidazole and 20% glycerol). The protein was eluted with two column volumes of elution buffer (50 mM HEPES/NaOH, pH 8, 18 mM CHAPS, 0.5 M NaCl, 250 mM imidazole and 20% glycerol). Imidazole was removed by gel filtration (Econo-Pac 10DG Columns Bio-Rad), and the purified protein was quickly frozen and stored in liquid nitrogen. Protein concentration was determined before freezing with Bio-Rad protein assay using bovine serum albumin as a standard. A dynamic light scattering apparatus (Zetasizer Nano, Malvern Instruments, Worcestershire, UK) was used to monitor the absence of aggregation of purified ABCG2 in detergent solution, either before freezing or after freezing and thawing, taking into account the viscosity and refraction index of the buffer. All steps were performed in the presence of 1,4-dithio-DL-threitol (5 mM) and protease inhibitors (1 µl cocktail/ml buffer).

**ATPase activity measurements.** The ATPase assay was based on a colorimetric ascorbic acid/ammonium molybdate assay for measuring the release of inorganic phosphate as described [34] with minor modifications. The reaction mixture contained 50 mM HEPES/NaOH, pH 8, 5 mM 1,4-dithio-DL-threitol, an ATP-regenerating system (4 mM phosphoenolpyruvate, 60 µg/ml pyruvate kinase), 0.5 µg of purified protein and 50 µg azolectin. The reaction was started by addition of 5 mM ATP/20 mM $MgCl_2$ and proceeded for 40 min at 37 °C. Orthovanadate was prepared as described [35]. Each experiment was performed two times in triplicate, and experimental data were analyzed with SigmaPlot.

**Fluorescence quenching by nucleotides and drugs.** Experiments were performed at 25 °C using a Photon Technology International Quanta Master I spectrofluori-

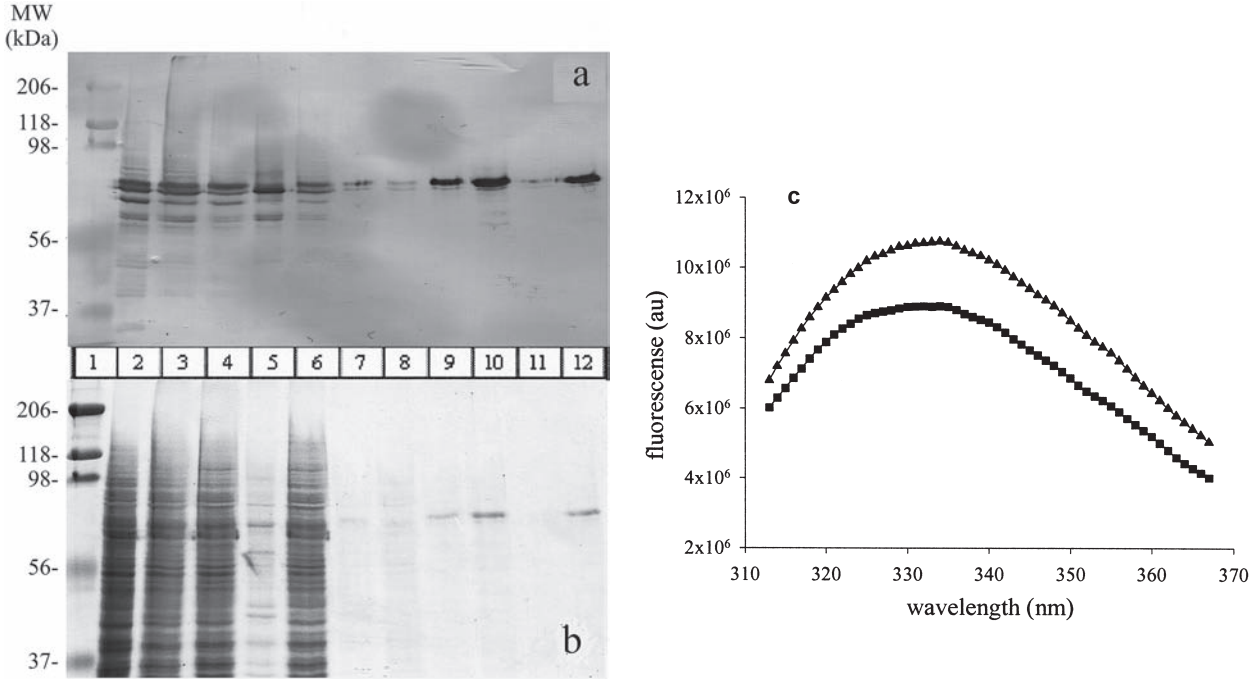


**Figure 1.** Purification of recombinant wild-type ABCG2. (*a*) Western blot revealed with anti-ABCG2 monoclonal antibody BXP-21. (*b*) SDS-PAGE Coomassie blue-stained gel. Lane 1: prestained molecular weight markers; lane 2: inverted membrane vesicles of High Five cells infected with baculovirus vector encoding R482 or R482T ABCG2; lane 3: supernatant after solubilization; lane 4: supernatant after centrifugation (15 000 *g*, 30 min); lane 5: detergent-insoluble pellet after centrifugation; lane 6: supernatant after binding for 2 h; lane 7: Ni-NTA agarose gel after binding for 2 h; lane 8: washing; lane 9: Ni-NTA agarose gel after washing; lane 10: elution; lane 11: Ni-NTA agarose gel after elution; lane 12: after imidazole removal by gel filtration. (*c*) Intrinsic fluorescence spectra. Buffer-corrected fluorescence emission spectra for purified wild-type R482 (■) or mutant R482T (▲) transporter at (0.045 mg protein/ml); the fluorescence emission spectra were recorded at 25 °C upon excitation at 295 nm. Representative spectra are shown here, similar results being obtained in many experiments from two different preparations of both wild-type and mutant transporters.

meter with spectral bandwidths of 4 nm. All measurements were corrected for buffer contribution to fluorescence, and inner-filter effects measured on *N*-acetyltryptophanamide. Purified protein (0.045 mg/ml into 50 mM HEPES/NaOH, pH 8, 18 mM CHAPS, 0.5 M NaCl and 20% glycerol) was excited at 295 nm and the emission spectra were scanned from 310 to 370 nm. The concentration-dependent quenching studies were performed at least three times, and the most representative curve was fitted with the Grafit program (Erithacus software). For nucleotides that produced a monophasic quenching curve the experimental data were fitted to the one-site ligand binding model, as previously described [36]. For substrate drugs and inhibitors that showed biphasic quenching curves, the experimental data were fitted according to a two-site model as described by Doppenschmitt et al. [37].

## Results

**Overexpression of human ABCG2 in insect cells, solubilization and purification.** The cDNA encoding the hexahistidine-tagged wild-type transporter was recombined with baculovirus, and used to infect High Five insect cells, and cell culture was harvested after 72 h for membrane preparation. The fractions obtained from solubilization with 1.1% CHAPS and purification with nickel-chelate affinity chromatography (Fig. 1) were analyzed by SDS-PAGE, and followed by Western blot with the specific BXP-21 monoclonal antibody (Fig. 1a) and Coomassie blue staining (Fig. 1b). The transporter appeared to be efficiently overexpressed in the membrane fraction (lane 2) and recovered upon detergent solubilization (lane 4). A good purification was achieved through

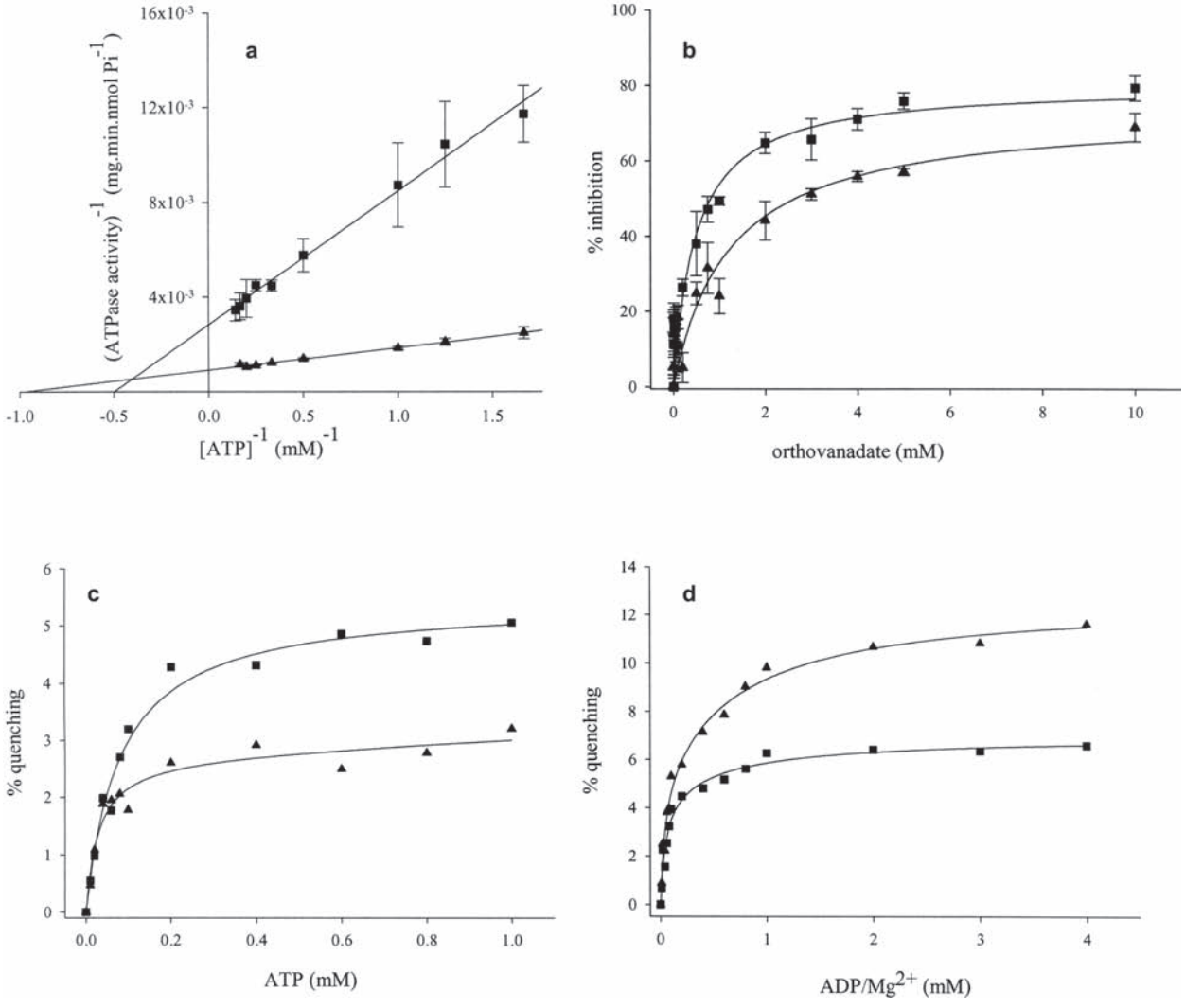


**Figure 2.** ATPase activity and nucleotide binding. (*a*) Lineweaver-Burk plots of ATP hydrolysis by the purified transporters. The ATPase activity of purified R482 (■) or R482T (▲) ABCG2 was measured with 0.5 µg protein in the presence of 50 µg azolectin at 37 °C for 40 min. (*b*) Inhibition by orthovanadate of ATPase activity. The inhibition of ATPase activity of R482 (■) and R482T transporter (▲) in the presence of 5 mM MgATP was measured at increasing orthovanadate concentrations (0.001–10 mM). (*c*, *d*) nucleotide binding as monitored by quenching of the intrinsic tryptophan fluorescence of purified R482 (■) and R482T (▲) transporter at 0.045 mg/ml: ATP (*c*) and ADP/$Mg^{2+}$ (*d*). Fluorescence emission was recorded from 310 to 370 nm at 25 °C upon excitation at 295 nm.

**Table 1.** Binding parameters for quenching of ABCG2 intrinsic fluorescence by nucleotides and drugs. The concentration-dependent saturable curves obtained in Figs 2–4 with wild-type and mutant purified ABCG2 were fitted by the Grafit program, according to a one-site model for nucleotides [29], or a two-site model for substrate drugs and inhibitors [30]. Apparent dissociation constant and maximal quenching values are indicated. For the two-site model, the program gave $\Delta F_1$ and $\Delta F_{max}$. ΔF2 was then calculated by the difference between $\Delta F_{max}$ and $\Delta F_1$.

| | Wild-type R482 | | | | Mutant R482T | | | |
|---|---|---|---|---|---|---|---|---|
| | $K_{D1}$ (μM) | $\Delta F_1$ (%) | $K_{D2}$ (μM) | $\Delta F_{max}$ (%) | $K_{D1}$ (μM) | $\Delta F_1$ (%) | $K_{D2}$ (μM) | $\Delta F_{max}$ (%) |
| *Nucleotides* | | | | | | | | |
| ATP | 81.3 ± 10.4 | 5.42 ± 0.19 | | | 36.1 ± 7.4 | 2.99 ± 0.14 | | |
| MgADP | 82.0 ± 13.5 | 6.42 ± 0.23 | | | 149 ± 22 | 11.1 ± 0.4 | | |
| *Substrate drugs* | | | | | | | | |
| Methotrexate | 4.48 ± 0.40 | 13.7 ± 3.4 | 84.2 ± 25.6 | 66.3 ± 3.4 | 7.30 ± 0.57 | 15.7 ± 4.7 | 87.0 ± 29.4 | 84.3 ± 4.7 |
| Rhodamine 123 | 0.52 ± 0.10 | 5.66 ± 1.21 | 16.6 ± 6.6 | 24.4 ± 1.2 | 0.42 ± 0.09 | 6.11 ± 1.23 | 15.4 ± 6.6 | 23.9 ± 1.2 |
| Doxorubicin | 1.63 ± 0.45 | 12.4 ± 3.4 | 22.2 ± 11.1 | 47.6 ± 3.4 | 2.91 ± 0.60 | 7.32 ± 2.36 | 25.9 ± 9.0 | 40.7 ± 2.4 |
| Mitoxantrone | 0.65 ± 0.06 | 4.03 ± 0.42 | 20.3 ± 4.3 | 16.0 ± 0.4 | 0.80 ± 0.10 | 4.53 ± 0.10 | 17.8 ± 4.6 | 25.5 ± 0.1 |
| Pheophorbide a | 0.94 ± 0.12 | 10.0 ± 1.3 | 17.7 ± 4.2 | 40.0 ± 1.3 | 1.50 ± 0.23 | 6.77 ± 1.75 | 21.7 ± 6.3 | 38.2 ± 1.8 |
| Hoechst33342 | 2.04 ± 0.67 | 12.8 ± 2.3 | 28.7 ± 19.6 | 32.2 ± 2.3 | 1.69 ± 0.40 | 14.0 ± 4.1 | 23.1 ± 9.6 | 66.0 ± 4.1 |
| *Inhibitors* | | | | | | | | |
| GF120918 | 2.71 ± 0.66 | 12.6 ± 5.1 | 25.7 ± 10.9 | 72.4 ± 5.1 | 6.23 ± 1.46 | 9.6 ± 5.2 | 30.9 ± 11.7 | 70.9 ± 5.2 |
| 6-prenylchrysin | 0.80 ± 0.11 | 8.22 ± 2.39 | 26.8 ± 9.2 | 51.8 ± 2.4 | 0.57 ± 0.07 | 11.0 ± 2.7 | 23.4 ± 6.1 | 69.0 ± 2.7 |
| Ko143 | 0.012 ± 0.0016 | 13.2 ± 7.9 | 0.14 ± 0.11 | 66.8 ± 7.9 | 0.012 ± 0.0005 | 8.63 ± 3.63 | 0.096 ± 0.02 | 91.4 ± 3.6 |
| Novobiocin | 2.56 ± 0.56 | 4.45 ± 2.07 | 23.8 ± 10.8 | 27.6 ± 2.1 | 1.49 ± 0.33 | 8.75 ± 2.87 | 21.1 ± 10.0 | 41.3 ± 2.9 |

nickel binding (lane 7), washing with 30 mM imidazole (lane 9), elution by 250 mM imidazole (lane 10) and removal of imidazole (lane 12). The typical amount of purified ABCG2 was 0.7 mg protein from 55 mg total membrane proteins, corresponding to a 1.3% yield, as prepared from four 225-$cm^2$ flasks of cultured insect cells. A significantly lower yield was obtained in Sf9, as compared with High Five, insect cells as due to lower overexpression (not shown here). A similar procedure was used to overexpress, solubilize and purify mutant R482T ABCG2: qualitatively similar results were obtained as in Fig. 1a and b, but with a twofold lower yield (data not shown). Both wild-type and mutant transporters exhibited a tryptophan-characteristic emission spectrum of intrinsic fluorescence, with a maximal emission wavelength around 333 nm upon excitation at 295 nm (Fig. 1c), indicating a rather hydrophobic average environment around the tryptophan residues. It is worthwhile mentioning that the R482T point mutation significantly increased fluorescence intensity, by nearly 20%, giving rise to a more symmetrical emission spectrum.

**ATPase activity and nucleotide binding.** Both transporters exhibited a high ATPase activity upon addition of lipids. The maximal rate of wild-type ABCG2 was 357 ± 72 nmol ATP hydrolyzed/min/mg, with a $K_m$(MgATP) of 2.0 ± 0.4 mM (Fig. 2a). Interestingly, the R482T point mutation both increased $V_{max}$, up to 1111 ± 79 nmol ATP hydrolyzed/min/mg, and lowered the $K_m$(MgATP) to 1.0 ± 0.1 mM. Concomitantly, the strong inhibition by orthovanadate (80 ± 0.5%) became 2.5-fold less sensitive towards concentration when comparing the $IC_{50}$ values (Fig. 2b). Even without added lipids, the purified recombinant protein was able to bind nucleotides, as monitored by quenching of intrinsic fluorescence. Saturable uni-site curves were obtained as a function of increasing concentrations for both ATP (Fig. 2c) and ADP (Fig. 2d). Interestingly, the R482T mutation increased the binding affinity for ATP (in the absence of magnesium ions to avoid hydrolysis), with a shift in apparent $K_D$ from 81.3 ± 10.4 to 36.1 ± 7.4 μM, whereas it lowered the binding affinity for MgADP, the hydrolysis product, with a shift in $K_D$ from 82.0 ± 13.5 to 149 ± 22 μM (*cf.* Table 1).

**Direct interaction of ABCG2 with substrate drugs.** A number of compounds known to be transported by ABCG2 were found here to produce a saturable concentration-dependent quenching of intrinsic fluorescence of the purified transporter (Fig. 3). However, curve fitting indicated two binding sites for each substrate drug. For methotrexate, the apparent $K_{D1}$ and $K_{D2}$ values for wild-type ABCG2 were 4.48 ± 0.40 and 84.2 ± 25.6 μM, respectively (Fig. 3a, and Table 1). Interestingly, the R482T point mutation, reported to abolish methotrexate transport by whole cells and inverted membrane vesicles, was found here to only slightly alter $K_{D1}$ (7.30 ± 0.57 μM), while $K_{D2}$ remained unchanged (87.0 ± 29.4 μM), whereas maximal quenching was higher for mutant as compared with wild-type ABCG2. Conversely, although wild-type ABCG2 was known not to transport rhodamine 123, in contrast to R482T mutant [8], displaying here apparent $K_{D1}$ and $K_{D2}$ values of 0.52 ± 0.10 and 16.6 ± 6.6 μM, it in fact bound the fluo-

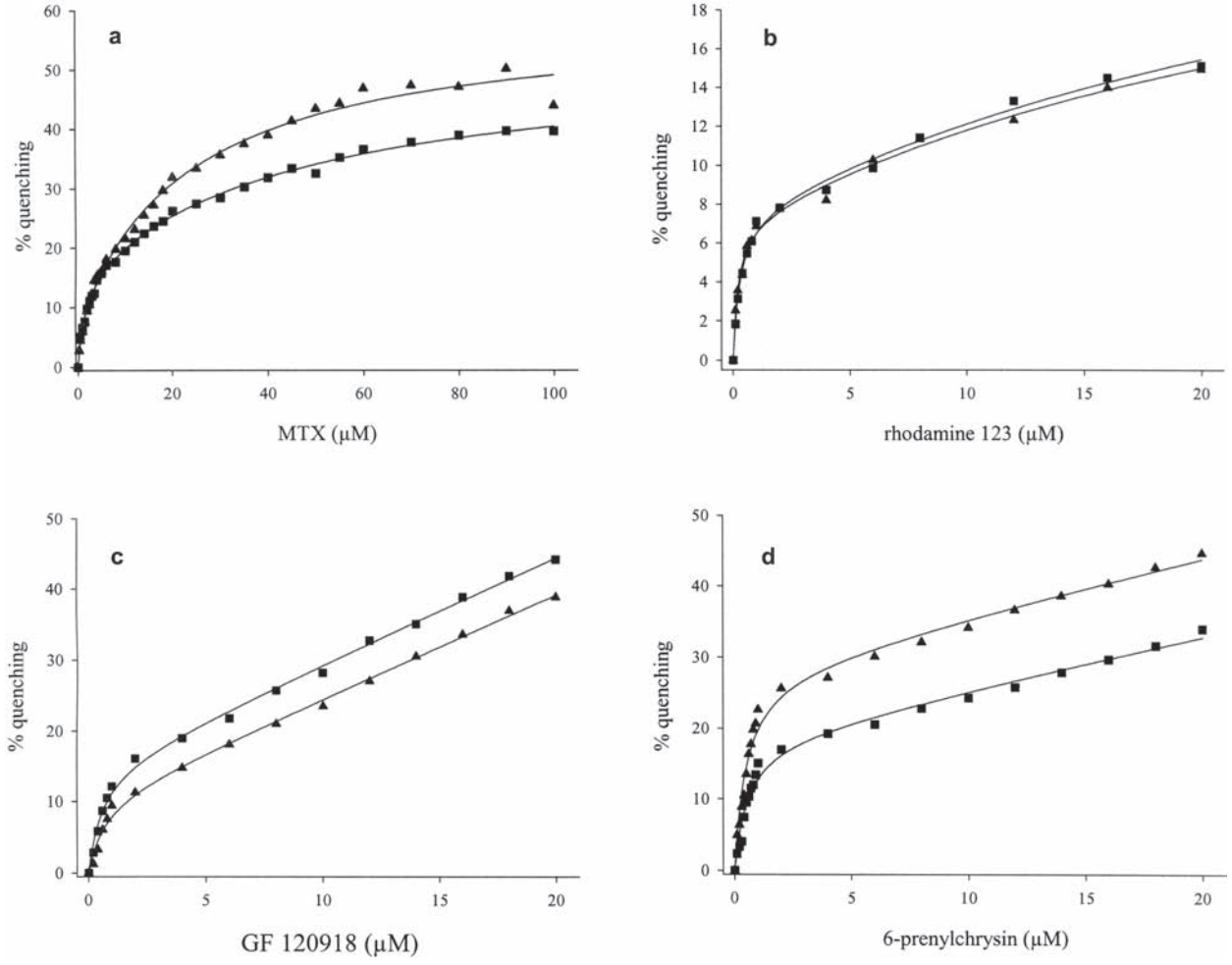


**Figure 3.** Quenching of the intrinsic fluorescence of purified ABCG2 by substrate drugs and inhibitors. Fluorescence emission was recorded, as in Fig. 2c, d for R482 (■) and R482T (▲) transporters, in the presence of increasing concentrations of either substrate drugs such as methotrexate (*a*) and rhodamine 123 (*b*), or known inhibitors such as GF120918 (*c*) and 6-prenylchrysin (*d*).

rescent dye similarly (0.42 ± 0.09 and 15.4 ± 6.6 μM, respectively) (Fig. 3b, and Table 1). Furthermore, doxorubicin and mitoxantrone are known to be transported much better by mutant as compared with wild-type transporter; the binding curves again showed that the apparent $K_D$ values, 1.63 ± 0.45 and 22.2 ± 11.1 μM for doxorubicin, or 0.65 ± 0.06 and 20.3 ± 4.3 μM for mitoxantrone, were not markedly altered, if at all, by the mutation, leading to the respective values of 2.91 ± 0.60 and 25.9 ± 9.0 μM for doxorubicin and 0.80 ± 0.10 and 17.8 ± 4.6 μM for mitoxantrone (Table 1). Two other substrates were assayed, which bound similarly to wild-type and mutant purified ABCG2: firstly, the natural substrate pheophorbide a with $K_D$ values of 0.94 ± 0.12 and 17.7 ± 4.2 μM compared with 1.50 ± 0.23 and 21.7 ± 6.3 μM and, secondly, the fluorescent dye Hoechst33342 with $K_D$ values of 2.04 ± 0.67 and 28.7 ± 19.6 μM compared with 1.69 ± 0.40 and 23.1 ± 9.6 μM (Table 1). As a control, glutathione, a charged substrate of MRP1 but not of ABCG2, did not significantly modify the intrinsic fluorescence of purified wild-type transporter, up to millimolar concentrations.

**Binding of known inhibitors.** GF120918 and 6-prenylchrysin have been recently shown to lead to a better inhibition (22-fold and 12-fold, respectively) of the mitoxantrone efflux catalyzed by wild-type as compared with mutant ABCG2-transfected HEK cells, as measured by flow cytometry [12]. In contrast, the present binding experiments (Fig. 3c and d, Table 1) displayed only very limited changes, if any: 2.71 ± 0.66 and 25.7 ± 10.9 μM as compared with 6.23 ± 1.46 and 30.9 ± 11.7 μM for GF120918 on the one hand, 0.80 ± 0.11 and 26.8 ± 9.2 as compared with 0.57 ± 0.07 and 23.4 ± 6.1 for 6-prenylchrysin on the other hand. Interestingly, a much higher binding affinity was observed for Ko143 ($K_{D1}$ of 12 ± 1.6 nM or 12 ± 0.5 nM for wild-type or mutant ABCG2, respectively, Table 1), which is known as a second-generation highly potent inhibitor of both drug trans-

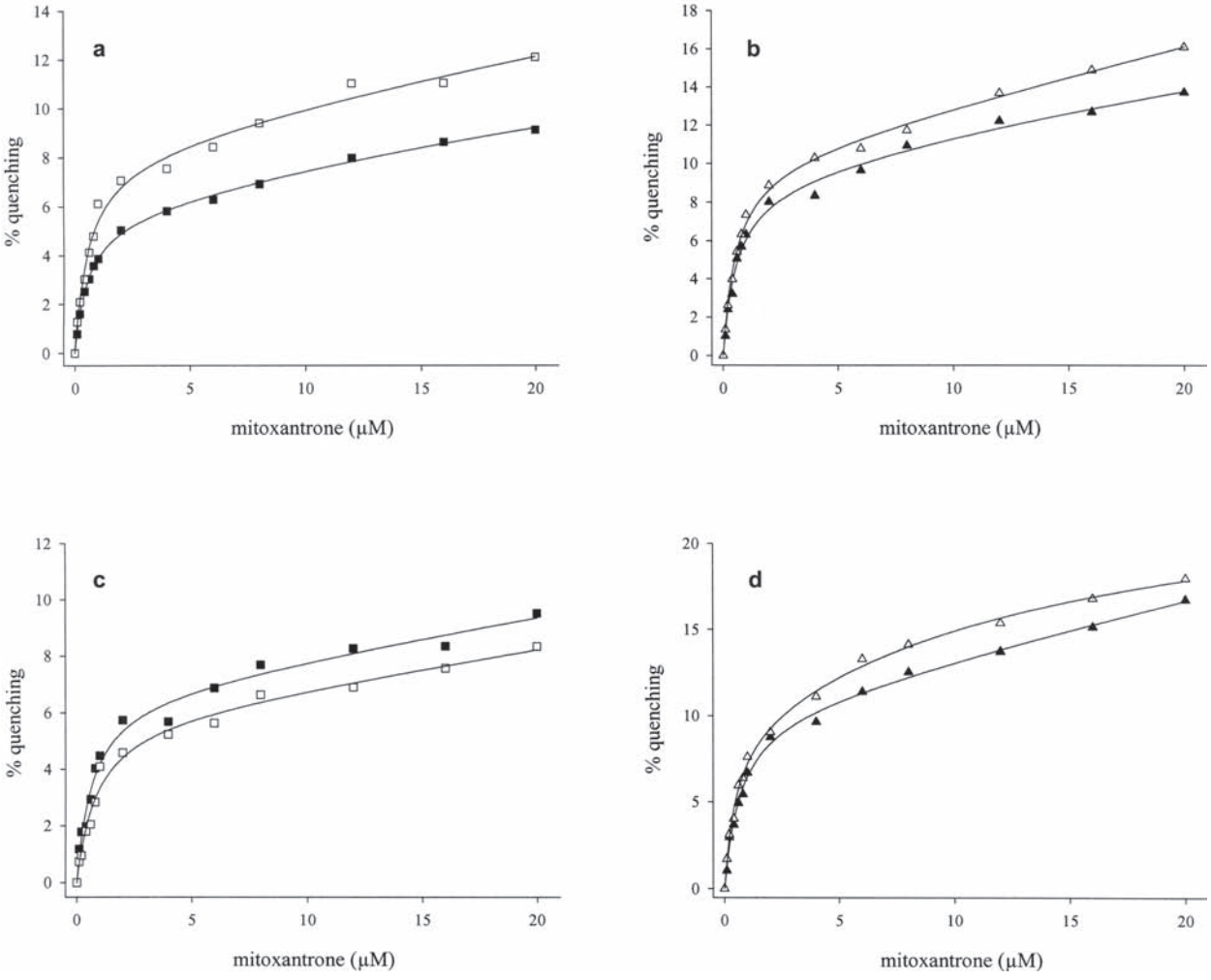


**Figure 4.** Prevention by inhibitors of mitoxantrone binding. Wild-type (*a*, *c*) or mutant (*b*, *d*) ABCG2 was preincubated for 30 min with either 5 μM of GF120918 (*a*, *b*) or 3 μM 6-prenylchrysin (*c*, *d*) (open symbols) or without inhibitor (closed symbols), and the concentration-dependent binding of mitoxantrone was then measured under conditions of Fig. 3d.

port and ATPase activity [11, 31]. Finally, the lower affinity inhibitor novobiocin also bound similarly to wild-type and mutant transporter, with $K_D$ values of 2.56 ± 0.56 and 23.8 ± 10.8 μM as compared with 1.49 ± 0.33 and 21.1 ± 10.0 μM (Table 1).

**Effects of preincubation with inhibitors on mitoxantrone binding.** The differential inhibitory effects of GF120918 and 6-prenylchrysin observed on mitoxantrone efflux [12] might relate to either its binding, or its transport, or both. Figure 4 clearly shows that preincubation of either wild-type or mutant ABCG2 with each inhibitor did not significantly modify further mitoxantrone binding. Indeed, similar saturable quenching curves were obtained for wild-type ABCG2 whether GF120918 was present (0.56 ± 0.08 and 17.3 ± 4.6 μM) or not (0.65 ± 0.06 and 20.3 ± 4.3 μM) (Fig. 4a). Similar results were obtained with the mutant transporter: 0.61 ± 0.07 and 19.0 ± 4.6 μM as compared with 0.80 ± 0.10 and 17.8 ± 4.6 μM (Fig. 4b). A comparable behavior was observed for 6-prenylchrysin with both wild-type ABCG2 (0.87 ± 0.23 and 28.6 ± 22.6 μM, Fig. 4c) and the mutant transporter (0.74 ± 0.11 and 17.8 ± 8.8 μM, Fig. 4d).

## Discussion

This paper reports a first purification of human breast cancer resistance protein ABCG2, enabling measurement of direct interactions with substrates and inhibitors by taking advantage of a hydrophobic tryptophan environment highly sensitive to ligand binding; this allowed a molecular characterization of the role of arginine-482 known to control the spectrum of transported drugs and the related cellular multidrug resistance.

**Heterologous overexpression and purification of functional wild-type or mutant ABCG2.** The ability of

baculovirus-infected insect cells to produce recombinant active human ABCG2 was clearly demonstrated by retention of drug-efflux activity of cells before lysis, as well as ATP hydrolysis and transport capacity of inverted vesicles of enriched membranes [12, 24, 25]. It is shown here that reasonable amounts of purified ABCG2 can be obtained upon solubilization with CHAPS and one-step affinity chromatography, allowing biochemical and biophysical studies. At least some of the five tryptophan residues per monomer appear to be strategically located, within a rather hydrophobic environment, since the interaction with all assayed known substrates and inhibitors produced a significant quenching of intrinsic fluorescence. Such concentration-dependent modifications were quite convenient to compare the differential effects produced by substrates and inhibitors, and the different properties of wild-type and mutant transporters. The R482T mutant ABCG2 could be also prepared by the same procedure despite a twofold lower yield that might indicate some loss in protein stability. The mutation seems to induce some local structural change as deduced from the differences observed in both the intensity of the emission fluorescence spectrum (Fig. 1) and maximal quenching values induced by various ligands (Figs. 2–4), whereas the apparent dissociation constants were only slightly, if at all, modified. The functionality of both recombinant purified transporters was assessed by (i) the high ATPase activity, sensitive to vanadate and dependent on the R482T mutation, (ii) the direct binding of nucleotides, in the presence or absence of magnesium ions and their dependence on the mutation, (iii) the binding of most substrate drugs and inhibitors in the micromolar range, consistent with their known cellular effects, and (iv) the very high-affinity binding, in the nanomolar range, of Ko143 recognized as the most efficient ABCG2 inhibitor.

**The R482T hot-spot mutation as a gain-of-function for ATP hydrolysis.** The high maximal rate of vanadate-sensitive ATPase activity and millimolar affinity for MgATP of purified ABCG2 are consistent with results previously obtained with enriched insect cell membranes [12, 24, 25], and overexpression of the transporter in the range of 1–3% of total membrane proteins within these membranes. The mutation-induced threefold increase in maximal rate of the purified transporter (up to 1.1 μmol ATP hydrolyzed/min/mg) is consistent with results of ABCG2-enriched membranes with various mutations of arginine-482, demonstrating a gain-of-function for ATP hydrolysis capacity [8, 24]. It is further shown here that the gain-of-function induced by the R482T mutation is also characterized by a twofold increase in affinity for MgATP, concomitant with a better binding of substrate ATP and a better release of product ADP. In addition, the lowered affinity for inhibitory vanadate suggests that, as for ADP, the release of product phosphate might be facilitated, and, therefore, might constitute a less limiting step in the ATPase reaction.

**Drug interaction appears to be independent of the ability to be transported and to induce cellular resistance.** By contrast to uni-site binding curves for nucleotides, all known substrate drugs and inhibitors assayed here bound according to biphasic curves as a function of concentration. This could be fitted as either a one-site model with additional nonspecific binding, or a two-site model [37]. The latter was used for P-glycoprotein for which similar binding curves were obtained, some of which with the same ligands [31]. Our results shown in Table 1 were therefore analyzed according to a two-site model. Of course, a one-site model with additional nonspecific binding could not be completely excluded; however, the absolute values of $K_{D2}$ were not of critical importance here, since the first aim was to compare mutant to wild-type transporters, for which nonspecific ligand binding, if any, should be similar. Moreover, the first, high-affinity, phase of binding characterized by $K_{D1}$ appeared quite relevant to specific drug binding and could be confidently used for studying the mutation effects. Depending on the substrate, the higher affinity constant, $K_{D1}$, was in the range 0.4–7 μM, whereas the lower affinity one, $K_{D2}$, was in the range 15–87 μM. A similar two-site binding of most substrate drugs, including rhodamine 123 and doxorubicin, was also reported with P-glycoprotein under similar conditions to those used here, contrasting with uni-site binding of nucleotides [31]. The substrate displaying the lowest affinity was methotrexate ($4.47 \pm 0.40$ and $82.4 \pm 26.6$ μM for wild-type purified ABCG2), consistent with the relatively high concentrations required for measuring a vesicular transport [38] as compared with other known ABCG2-transported drugs. These two binding sites for methotrexate are consistent with the positive cooperativity of transport recently reported [24]. Surprisingly, methotrexate bound nearly as efficiently to purified mutant ABCG2 ($7.30 \pm 0.57$ and $87.0 \pm 29.4$ μM) despite the absence of cellular [16] or vesicular transport [17, 24] by the latter. However, other recent reports suggested that methotrexate was also able to interact with mutant ABCG2 from its capacity to inhibit both photoaffinity labeling within enriched membranes by a rhodamine 123 derivative [39] and Hoechst33342 transport [40], and to induce a short-term resistance of cell growth [41].
Conversely, rhodamine 123 and doxorubicin are known to be effluxed and to induce cellular resistance in the case of mutant, but not of wild-type, transporter [18, 19, 21]. The present results show that the two drugs bound to purified wild-type ABCG2 and to the mutant transporter, in agreement with photoaffinity labeling of both transporter variants by an iodoaryl-azido derivative of rhodamine 123, and prevention of the photolabeling by doxorubicin [39]. In addition, although mitoxantrone is also known to be better transported and to confer stronger resistance

to cells expressing mutant as compared with wild-type ABCG2 [21], it was shown here to bind equally to both purified transporters. The overall results strongly suggest that all drugs that are able to bind to wild-type ABCG2 also bind similarly to the mutant transporter, whether they are transported or not, and therefore whatever the residue at position 482.

**Mutation-independent binding of substrate drugs and inhibitors, suggesting a role of arginine-482 on drug transport.** In the case of Ko143, not only substrate drugs but also inhibitors bound similarly to both variants of purified ABCG2, with an extremely high affinity in the nanomolar range, consistent with its strong potency to inhibit both drug transport and related cell growth resistance, as well as ATPase activity [11, 42]. Although novobiocin [21], on the one hand, and GF120918 and 6-prenylchrysin [12], on the other hand, have been shown to inhibit the efflux of BODIPY-prazosin and mitoxantrone, respectively, much more efficiently, in cells transfected by wild-type as compared with mutant *ABCG2*, they bound here similarly to both variants of the purified transporter. This clearly indicates that the differential inhibitory effects observed when comparing wild-type to mutant ABCG2-transfected cells were not due to differences in affinity of inhibitor binding, but more likely to the differences in substrate drug transport, as discussed above. Such a lack of effect of a single point-mutation on drug binding is not so surprising for a multispecific transporter involved in cell detoxification. It was previously characterized as having a wide spectrum for drug binding, as shown by the ability of many compounds to antagonize photoaffinity labeling of the rhodamine 123 binding site [39] within enriched membranes. The $K_{D1}$ values obtained here for inhibitors correlate well with previously reported $IC_{50}$ values, *e.g.* in the 5–10 nM range for Ko143 towards ATPase activity, Hoechst33342 transport and sensitization to mitoxantrone [42], or the submicromolar range for 6-prenylchrysin toward mitoxantrone transport and sensitization to mitoxantrone [12].The micromolar range obtained for novobiocin binding confirms problems of cellular accessibility [21] likely due to low membrane penetration [13]. Comparable binding for substrates and inhibitors were obtained with P-glycoprotein [31], with monophasic binding curves for nucleotides and biphasic ones for most substrate drugs and inhibitors. Rhodamine also bound in the micromolar range, as did a number of other drugs.

The present lack of prevention of mitoxantrone binding to purified ABCG2 by preincubation with either GF120918 or 6-prenylchrysin suggests essentially distinct binding sites for substrate drug and inhibitors. This was indeed also proposed in the case of P-glycoprotein, by considering either rhodamine 123 and Hoechst33342 substrate sites towards prazosin and progesterone regulatory sites [43], or vinblastine, paclitaxel and Hoechst33342 substrate sites toward GF120918 and nicardipine regulatory sites [44]. An alternative explanation would be that GF120918 and 6-prenylchrysin bind to another substrate drug site, the saturation of which by a different substrate does not antagonize mitoxantrone transport, as reported in *Xenopus laevis* oocytes for rhodamine 123, topotecan and daunorubicine against mitoxantrone, or for any tested substrate against flavopiridol [45]. A similar lack of antagonism between several drug substrates was also reported for P-glycoprotein [46].

In conclusion, the R482T mutation, which occurs *in vitro* on cell cultures submitted to high drug pressure, induces a gain-of-function for ATPase activity and for substrate-drug transport, but not drug binding. The expected location of arginine-482 within the third transmembrane span of the ABCG2 transmembrane domain and the drastically different effects on various substrate drugs suggest a direct role on drug transport. By contrast, the stimulation of ATPase activity might be related to distant, indirect, effects mediated through conformational changes possibly associated to energy coupling between ATP hydrolysis and drug transport. The absence of mutation at this position *in vivo,* despite a number of reported polymorphism examples [47], might indicate deleterious effects produced by altered transport selectivity of natural substrates. Such a critical role played by arginine-482 in transport control is consistent with the residue conservation during evolution.

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