ABCG2 (Breast Cancer Resistance Protein/Mitoxantrone Resistance-Associated Protein) ATPase Assay: A Useful Tool to Detect Drug-Transporter Interactions

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Received December 28, 2006; accepted May 24, 2007

Abstract:

The ATPase assay using membrane preparations from recombinant baculovirus-infected Spodoptera frugiperda ovarian (Sf9) cells is widely used to detect the interaction of compounds with different ATP-binding cassette transporters. However, Sf9 membrane preparations containing the wild-type ABCG2 transporter show an elevated baseline vanadate-sensitive ATPase activity, which cannot be further stimulated by substrates of ABCG2. Therefore, this assay system cannot be used for the detection of ABCG2 substrates. To overcome this difficulty we 1) purified membranes from a selected human cell line expressing wild-type ABCG2, and 2) inhibited the baseline ATPase activity with different inhibitors. In our modified assay, ABCG2 substrates were able to stimulate the baseline ATPase activity of ABCG2 expressed in membranes of human cells. Furthermore, using the specific ABCG2 inhibitors Ko143 or Ko134 allowed us to suppress the baseline vanadate-sensitive ATPase activity. Substrates of ABCG2 could stimulate this suppressed baseline ATPase, resulting in a better signal-to-background ratio and a robust assay to detect substrates of the ABCG2 transporter. The ATPase assay and the direct vesicular transport measurements for estrone-3-sulfate were in good accordance.

ABCG2 [breast cancer resistance protein (BCRP), mitoxantrone resistance-associated protein (MXR), placenta-specific ATP-binding cassette (ABC) transporter] is an important factor in the pharmacokinetic properties of drugs and drug candidates (reviewed in Dietrich et al., 2003; Sarkadi et al., 2004). It is an ABC transporter known to be responsible for certain cases of multidrug resistance (reviewed in Litman et al., 2001). It is present in most of the pharmacologically relevant barriers of the human body. Inhibition of ABCG2 activity using specific inhibitors was shown to influence the pharmacokinetic properties of substrates of ABCG2 in mice (Allen et al., 2002) and humans (Kruijter et al., 2002; van Herwaarden and Schinkel, 2006). ABCG2 is also present in Caco-2 cells, which are widely used for permeability screening and drug-transporter interaction studies focusing on ABCB1 [P-glycoprotein (P-gp)] (Xia et al., 2005). Altogether, the central modulating effect of the transporter on the pharmacokinetic parameters of certain drugs indicates that it is important to test the interaction of drug candidates with ABCG2 at the early phase of drug development.

The ATPase assay using Spodoptera frugiperda ovarian (Sf9) cell membrane preparations is a widely used assay to test the interaction of compounds with different transporters, including ABCB1/P-gp/MDR1 and members of the ABCC/multidrug resistance-associated protein subfamily (Sarkadi et al., 1992; Bakos et al., 2000; Bodo et al., 2003). It is a relatively inexpensive screening tool, which, besides detecting the interaction of test drugs and ABC transporters, may also indicate the nature of the interaction. Other available tools have the disadvantage of lower throughput and higher cost (monolayer studies, direct vesicular transport), or they provide less information on the nature of the interaction (inhibition studies).

ABBREVIATIONS: BCRP, breast cancer resistance protein; MXR, mitoxantrone resistance-associated protein; ABC, ATP-binding cassette; P-gp, P-glycoprotein; Sf9 cells, Spodoptera frugiperda ovarian cells; GF120918, N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)(ethyl)-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; MOPS, 3-(N-morpholino)propanesulfonic acid; MS, mass spectrometry.
The first ABCG2 ATPase assays using S9 membranes were reported on the R482G version of the transporter (Ozvegy et al., 2001). This membrane preparation had very high baseline vanadate-sensitive ATPase activity, which could be stimulated by a number of known ABCG2 substrates. Later studies showed that a single amino acid change resulted in significant changes in the substrate specificity of this protein, highlighting the importance of amino acid 482 in substrate binding and/or transport activity of ABCG2 (Honjo et al., 2001; Ozvegy et al., 2002; Ozvegy-Laczka et al., 2005). S9 membranes containing the wild-type (482R) version of the transporter also exhibit high baseline vanadate-sensitive ATPase activity, which cannot be further stimulated by known BCRP substrates (Ozvegy et al., 2002; Ozvegy-Laczka et al., 2004). It was proposed that the different glycosylation pattern and/or the different membrane composition of the S9 cells could be responsible for this phenomenon (Ozvegy et al., 2001).

Membranes prepared from human expression systems have also been widely used in the field of ABC transporters (Loe et al., 1996; Sharom et al., 1999; Hirohashi et al., 2000). Unfortunately, these expression systems usually yield significantly lower expression levels that are insufficient to measure the ATPase activity of the transporter. Human cell lines selected with cytotoxic substrates of ABCG2 overexpress different variants of ABCG2 (Litman et al., 2000; Rocchi et al., 2000; Volk et al., 2000, 2002; Robey et al., 2001). According to our hypothesis, membranes prepared from these cell lines may be suitable for ATPase assay. Furthermore, the different glycosylation pattern and/or membrane composition may make these membrane preparations suitable for the detection of substrates of ABCG2 in the ATPase assay.

Several high affinity inhibitors (e.g., Ko143) of ABCG2 have been reported (Allen et al., 2002; Ozvegy et al., 2002; Ozvegy-Laczka et al., 2004; Xia et al., 2005). Our second hypothesis was that the baseline vanadate-sensitive ATPase activity of the membrane preparations might be suppressed by the addition of the above inhibitors, resulting in better signal-to-background ratios.

Materials and Methods

Chemicals. [3H]Methotrexate was purchased from Moravek Biochemicals (Brea, CA). [3H] Estrone-3-sulfate was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). GF120918 was synthesized at Altana Pharma Analytical Sciences (Boston, MA). Topotecan was purchased from LKT Laboratories (St. Paul, MN). GF120918 was synthesized at Altana Pharma Analytical Sciences (Boston, MA). [3H] Estrone-3-sulfate was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

Cell Culture and Membrane Preparation. All the ABCG2 preparations contained the wild-type (482R) version of the ABCG2 transporter (Accession number NM_004827). Human membrane vesicle preparations containing ABCG2 (MXR-M) and control human membrane preparations (M-CTRL), as well as insect cell membranes containing the human transporter (MXR-S9) and control insect membranes (β-gal-S9-CTRL, MXR-K86M-S9-CTRL), were obtained from SOLVO Biotechnology (Budapest, Hungary, http://www.solvo.com). The insect membrane vesicle preparations were obtained using recombinant baculoviruses encoding wild-type human ABCG2, inactive ABCG2-K86M mutant (carrying a mutation at a crucial position of the catalytic center of ATP binding and cleavage), and β-galactosidase (Ozvegy et al., 2001, 2002). S9 cells were cultured and infected with recombinant baculoviruses stocks as described earlier (Sarkadi et al., 1992). Purified membrane vesicles from virus-infected S9 cells were prepared essentially as described previously (Sarkadi et al., 1992). Membrane protein contents were determined using a modified Lowry procedure (Bensadoun and Weinstein, 1976).

Western Blotting. Protein expression was confirmed by SDS-polyacrylamide gel electrophoresis and subsequent Western blotting using specific anti-ABCG2 antibody BXP-21, horseradish peroxidase-conjugated anti-mouse secondary antibody (Sigma-Aldrich) and enhanced chemiluminescence (Amersham Biosciences) as described earlier (Ozvegy et al., 2002).

ATPase Assay. ATPase activity was measured as described earlier (Sarkadi et al., 1992). In brief, membrane vesicles (20 μg/well) were incubated in 10 mM MgCl2, 40 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-Tris, pH 7.0, 50 mM KCl, 5 mM dithiothreitol, 0.1 mM EGTA, 4 mM sodium azide, 1 mM ouabain, 5 mM ATP, and various concentrations of test drugs with or without 1.2 mM sodium orthovanadate for 40 min at 37°C. ATPase activities were determined as the difference of inorganic phosphate liberation measured in the presence or absence of 1.2 mM sodium orthovanadate (vanadate-sensitive ATPase activity). Results are presented as vanadate-sensitive ATPase activities or relative activities (%), where 100% is the baseline vanadate-sensitive ATPase activity of the membrane suspension.

Vesicular Transport Assay. Vesicular transport studies were performed as described (Bodo et al., 2003). Briefly, membrane fraction containing inside-out membrane vesicles was incubated in the presence or absence of 4 mM ATP in a buffer containing 7.5 mM MgCl2, 40 mM MOPS-Tris, pH 7.0, and 70 mM KCl at 37°C in the presence of the indicated substrate and other compounds for the indicated times. The transport was stopped by addition of 1 ml of cold wash buffer (40 mM MOPS-Tris, pH 7.0, 70 mM KCl) to the membrane suspensions and then rapidly filtered through class F glass fiber filters (pore size, 0.7 μm). Filters were washed with 2 × 5 ml of ice-cold wash buffer. When labeled compounds were used as substrates, radioactivity retained on the filter was measured by liquid scintillation counting. For unlabeled compounds, filters were processed as described under Analytics. ATP-dependent transport was calculated by subtracting the values obtained in the presence of AMP from those in the presence of ATP.

Analytes. Estradiol-3,17-disulfate. Samples were subjected to sort isotropic high-performance liquid chromatography separation before mass spectrometric (MS) analysis. High-performance liquid chromatography apparatus comprised the Applied Biosystems 140C (Foster City, CA), and the mobile phases were methanol/water in a volume ratio of 50:50%, flow rate 250 μl/min, sample volume 20 μl, column Hypersil, and 5 ODS, 2 × 2 mm. The samples were diluted in double-distilled water.

MS measurements were obtained on a Finnigian TSQ-7000 triple quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with a Finnigan Electrospray Ionization source. The instrument was operated in negative ion mode using selective reaction monitoring. In selective reaction monitoring mode, the first quadrupole was set to select 215 m/z ([M-H]+)2 of estradiol-3,17-disulfate, which was fragmented in the collision cell, and the most intense fragment at 175 m/z (the double-charged fragment ion showed a loss of H2SO3) was measured with the last quadrupole.

The electrospray ionization needle was adjusted to 4.5 kV, and N2 was used as a nebulizer gas. The collision potential was 25 eV. Argon was used as the collision gas, and the pressure in the collision cell region was 2 mtorr.

Chlorothiazide. An Agilent 1100 series liquid chromatography/diode array detector system (Agilent Technologies, Santa Clara, CA) was used, which consisted of the following modules: vacuum degasser (G1311A), binary pump (G1312A), well plate autosampler (G1367A), well plate thermostat (G1330B), column compartment (G1316A), and diode array detector (G1315B). System control and data acquisition were made with the Agilent ChemStation Version B.02.01.03R2. The equipment is coupled with a single quadrupole mass selective detector (Agilent Technology, Wilmington, DE) equipped with a straight needle alignment, standard fluxes, and an electrospray ionization ion source G1956A.

The chromatographic separations were performed on a Mercury MS, Synergi Fusion-RP column (20 × 4 mm, particle size 2 μm, pore size 80A, Phenomenex, Torrance, CA) coupled with a Security Guard Fusion-RP C18 precolumn (4 × 3 mm i.d., Phenomenex). The mobile phase was a mixture of 0.05% acetic acid in MilliQ water and acetonitrile (90:10, v/v). The flow rate was 0.5 ml/min. The volume of injection was 20 μl. The detector operated at a wavelength of 278 nm. The run time was 5 min.

The mass spectrometer was operated in negative ionization mode with a capillary voltage of 3 kV and cone voltage of 70 V. The temperature of source was maintained at 350°C. Nitrogen was used as the nebulizer gas, and it was set at flow rate of 12 l/h and pressure of 35 psi to generate the spray. Chlorothiazide was monitored in single ion mode at 294 m/z ([M-H]+).

Statistical Methods. Assays were run in duplicate, and unbiased (“n − 1”)
**FIG. 1.** A. ABCG2 levels in the different membrane preparations as detected by the monoclonal antibody BXP21 in Western blot. Ten micrograms of total membrane protein/sample was separated by 10% SDS-polyacrylamide gel electrophoresis and blotted on polyvinylidene difluoride membranes. The presence of ABCG2 was detected by the monoclonal antibody BXP-21 and anti-mouse/horseradish peroxidase secondary antibody visualized with enhanced chemiluminescence detection. Vanadate-sensitive baseline ATPase activity of MXR-Sf9 (B) and MXR-M (C) preparations in the presence of different ATP concentrations. Membranes containing 20 μg of total protein were incubated at 37°C for 40 min. Insert, Lineweaver-Burk plot. ATP-dependent [3H]methotrexate transport for MXR-Sf9 (D) and MXR-M (E) membrane vesicles at different methotrexate concentrations. Incubation time was the first linear phase of transport: 12 and 4 min for MXR-Sf9 membrane vesicles and MXR-M vesicles, respectively. Insert, Lineweaver-Burk plot. Inhibition of the ATP-dependent [3H]methotrexate transport (100 μM) for Sf9 and human membrane vesicles by different substrates and nonsubstrates (100 μM) and the specific inhibitor Ko143 (1 μM) of ABCG2 (F).

[Insert image of Western blot, ATPase assay graphs, and Lineweaver-Burk plots with data points and error bars for each condition.]
S.D. was calculated. Cutoff values in screening were defined as Cutoff = Baseline + 3 · Me, where Me is the median of the unbiased \( (n - 1) \) S.D. of the duplicate measurements of the dataset.

Results

Correlations of ABCG2 Activity in Human (MXR-M) and SF9 (MXR-SF9) Membranes. To compare the ABCG2 content and glycosylation level of the membrane preparations we used Western blotting using BXP-21, an ABCG2-specific antibody (Fig. 1A). SF9 preparations containing the wild-type or the defective (K86M) transporter (MXR-SF9, lane 1; MXR-K86M-SF9-CTRL, lane 2) displayed a strong band with apparent molecular mass of 55 to 60 kDa. No band was detected in membrane preparations prepared from SF9 cells infected with the baculovirus containing -galactosidase gene (-gal-SF9-CTRL, lane 3). Membrane preparations from the selected human cell line exhibited a strong band with an apparent molecular mass of 70 kDa (MXR-M, lane 4) that was absent in membrane preparations obtained from the parental line (M-CTRL, lane 5).

To compare the MXR-M and the MXR-SF9 membrane preparation, we first determined the vanadate-sensitive ATPase activity of the membrane preparations at different ATP concentrations (Fig. 1, B and C). Both membrane preparations showed similar \( K_m \) values for ATP (2.0 and 2.2 mM for MXR-SF9 and MXR-M membranes, respectively). ATP-dependent \([H]\\text{methotrexate vesicular transport measurements (Fig. 1, D and E) revealed that } [H]\\text{methotrexate transport has similar } K_m \text{ values (3.9 and 3.6 mM for MXR-SF9 and MXR-M membranes, respectively). Transport could be inhibited by the specific ABCG2 inhibitor KOl143 (1 } \mu M) \text{ in both preparations. Furthermore, the same substrate specificity was determined in the vesicular transport assay using various ABCG2 substrates (100 } \mu M) \text{ as an inhibition of methotrexate transport (Fig. 1F). The transport of } [H]\\text{methotrexate could not be observed in SF9 membranes containing the K86M-}}\)
FIG. 3. Vanadate-sensitive ATPase activity plotted as relative activity (%) of MXR-Sf9 (A, C, E, G) and MXR-M (B, D, F, H) preparations in the presence of different concentrations of sulfasalazine (x-axis) and inhibitors [A and B, Ko143 (nM); C and D, Ko134 (nM); E and F, Hoechst 33342 (µM); and G and H, GF120918 (µM)]. Vanadate-sensitive ATPase activity plotted as relative activity (%) of the MXR-M membrane preparation in the presence of different concentrations of prazosin (I) or topotecan (J) and Ko134 (nM). Membranes containing 20 µg of total protein were incubated at 37°C for 40 min.
defective mutant version of ABCG2, or in membranes prepared from the unselected, ABCG2-nonoverexpressing human cell lines (data not shown).

**ABCG2 ATPase Activity in MXR-M and MXR-Sf9 Membranes: Effect of Substrates and Inhibitors.** ABCG2 substrates sulfasalazine, topotecan, and prazosin (Litman et al., 2000; van der Heijden et al., 2004) showed only a slight modulation of the baseline vanadate-sensitive ATPase activity in the presence of 100 nM Ko143 or 200 nM Ko134. Sf9 membranes expressing the defective (MXR-K86M-Sf9-CTRL) version of ABCG2 show similar baseline vanadate-sensitive ATPase activity to the MXR-M preparation. In contrast, GF120918 showed inhibition at lower concentrations, whereas it showed increasing activity of the MXR-M preparation. In contrast, GF120918 showed inhibition at lower concentrations, whereas it showed increasing activity of the MXR-M preparation. Ko143, Ko134, Hoechst 33342, and GF120918) inhibited the baseline vanadate-sensitive ATPase activity in the presence of 100 nM Ko143 or 200 nM Ko134. The inhibition by GF120918 was similar to Ko143 and Ko134, except that increasing concentrations of GF120918 caused a smaller shift in the apparent EC50 for the activating effect of sulfasalazine shifted to higher concentrations (for MXR-M membranes 1.5 and 9.6 μM in the presence of 0 and 80 nM Ko143, respectively), suggesting that these interactions are competitive. Because of this competitive interaction as the inhibitors were added at higher concentrations, the activating effect of sulfasalazine became evident for the MXR-Sf9 membranes. The inhibition by GF120918 was similar to Ko143 and Ko134, except that increasing concentrations of GF120918 caused a smaller shift in the apparent EC50 values than in the case of Ko143/Ko134 (Fig. 3, G and H). Inhibition by Hoechst 33342 could not be reversed even by using increased concentrations of sulfasalazine. The apparent EC50 for the activating effect of sulfasalazine shifted to higher concentrations (for MXR-M membranes 1.5 and 9.6 μM in the presence of 0 and 80 nM Ko143, respectively), suggesting that these interactions are competitive. Because of this competitive interaction as the inhibitors were added at higher concentrations, the activating effect of sulfasalazine became evident for the MXR-Sf9 membranes. Therefore, inhibition by Ko143/Ko134 and GF120918 appeared to be competitive, whereas inhibition by Hoechst 33342 did not.

The assay using ABCG2 in membranes derived from human cells, with or without inhibition with Ko134 or Ko143, showed the best results, so we ran the experiment using different concentrations of Ko134 and the other two reference activators. Increasing concentrations of Ko134 inhibited the baseline vanadate-sensitive ATPase activity as earlier; however, when looking at the activating effect of these two compounds, we did not see any shift in apparent EC50 values, suggesting that the interaction of these two compounds with Ko134 is not competitive (Fig. 3, I and J).

We screened a library of 30 compounds at 100 μM using both membrane preparations with and without 100 nM Ko143 (Table 1).
No interaction was detected with MXR-S9, yet using Ko143 allowed the detection of the known BCRP substrates sulfasalazine, topotecan, and prazosin. The MXR-M preparation also detected the three known substrates in the absence of inhibitors, whereas applying Ko143 increased the signal-to-background (fold activation) values up to more than 4-fold. No false-positive results were seen for these 30 compounds in any assay setup used.

When using the MXR-M membranes, we found that in addition to the above-mentioned known substrates, estradiol-3,17-disulfate and chlorothiazide showed significant stimulation of its vanadate-sensitive ATPase activity. This activation could also be detected in the presence of Ko143/Ko134 with higher signal-to-background ratios. The two compounds did not show significant and reproducible stimulation of the vanadate-sensitive ATPase activity of MXR-S9 membranes (data not shown). To confirm that estradiol-3,17-disulfate and chlorothiazide are indeed substrates of ABCG2, we showed ATP-dependent accumulation of both compounds in MXR-M membrane vesicles in the vesicular transport assay (Fig. 4).

### Effect of pH on the Vesicular Transport and ATPase Activity of ABCG2 in the MXR-M Membrane.

In a recent report (Breedveld et al., 2007), it was shown that pH influences the transport properties of ABCG2. We measured the vesicular transport of [3H]methotrexate at pH 5.5 and 7.0 using our MXR-M vesicles at different methotrexate concentrations (up to 3 mM). The rate of transport at lower concentrations was lower at pH 7.0 than at pH 5.5. At pH 7.0, transport rates showed only slight saturation with increasing concentrations of meth-

![Graph A](image1.png)

### FIG. 4. A, vanadate-sensitive ATPase activity plotted as relative activity (%) of MXR-M membrane preparations in the presence of different concentrations of estradiol-3,17-disulfate in the absence and presence of 200 nM Ko134. B, transport of estradiol-3,17-disulfate measured in the vesicular transport assay at three different concentrations in the absence and presence of 4 mM MgATP. *, below the limit of detection. C, vanadate-sensitive ATPase activity plotted as relative activity (%) of MXR-M membrane preparations in the presence of different concentrations of chlorothiazide in the absence and presence of 80 nM Ko143. D, transport of chlorothiazide measured in the vesicular transport assay at three different concentrations in the absence and presence of 4 mM MgATP.

![Graph B](image2.png)

### TABLE 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH 5.5 EC50 (μM)</th>
<th>Maximal -Fold Activation</th>
<th>pH 7.0 EC50 (μM)</th>
<th>Maximal -Fold Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfasalazine</td>
<td>0.6 ± 0.1</td>
<td>2.9 ± 0.6</td>
<td>0.6 ± 0.1</td>
<td>3.4 ± 1.0</td>
</tr>
<tr>
<td>Prazosin</td>
<td>4.5 ± 0.7</td>
<td>2.3 ± 0.6</td>
<td>2.5 ± 0.7</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>Topotecan</td>
<td>25 ± 7.1</td>
<td>2.5 ± 0.2</td>
<td>17.5 ± 3.5</td>
<td>2.4 ± 1.1</td>
</tr>
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EC50, the concentration of half-maximal stimulation of the compound tested.

The vanadate-sensitive ATPase activity of MXR-M membranes (data not shown). To confirm that estradiol-3,17-disulfate and chlorothiazide are indeed substrates of ABCG2, we showed ATP-dependent accumulation of both compounds in MXR-M membrane vesicles in the vesicular transport assay (Fig. 4).
otrexate, whereas it turned into a more prominent saturation at pH 5.5. In the ATPase assay we also detected stimulation of the vanadate-sensitive ATPase activity at lower methotrexate concentrations at pH 5.5, which turned into saturation at higher concentrations. At pH 7.0, significant stimulation of the vanadate-sensitive ATPase activity was only reached at higher methotrexate concentrations, and it could not be saturated. For estrone-3-sulfate, vesicular transport was saturable at both pH values with similar kinetics. The compound stimulated the vanadate-sensitive ATPase activity with similar kinetics at both pH values. Sulfasalazine, topotecan, and prazosin also displayed similar stimulation of the ABCG2 ATPase activity when using the MXR-M preparation at pH 5.5 or 7.0 (Table 2).

Estrone-3-Sulfate ATPase and Vesicular Transport. To compare the kinetic parameters of ATPase and vesicular transport we measured the vanadate-sensitive ATPase activity and the vesicular transport of the ABCG2 substrate estrone-3-sulfate at different estrone-3-sulfate and Ko134 concentrations. In the ATPase assay, estrone-3-sulfate stimulated the baseline ATPase activity with a \( K_m \) value of 22 \( \mu \)M. It also showed a competitive interaction with Ko134 similar to that of sulfasalazine (see Fig. 6, A and B). In the vesicular transport assay, estrone-3-sulfate was transported with a \( K_m \) of 7.8 \( \mu \)M, and Ko134 inhibition was also competitive (see Fig. 6, C and D).

Discussion

We performed a comparative analysis of wild-type ABCG2 in membrane preparations derived from baculovirus-transfected Sf9 cells and selected human cells (MXR-Sf9 and MXR-M, respectively). Both transporter preparations had similar affinity for ATP and \(^{3}H\)methotrexate. The MXR-M preparations showed significantly higher \( V_{\text{max}} \) for \(^{3}H\)methotrexate transport (Fig. 1, D and E). One of the possible reasons for this difference is the difference in vesicular content of the two preparations. Substrates and inhibitors of ABCG2 inhibited, whereas noninteracting drugs did not modulate the ATP-dependent \(^{3}H\)methotrexate transport in either membrane preparation (Fig. 1F). In contrast, in the ATPase assays the same set of compounds showed a strikingly different pattern. In case of MXR-Sf9 preparations, the baseline vanadate-sensitive ATPase activity could not be further stimulated by ABCG2 substrates, whereas stimulation was detected for all the substrates tested for MXR-M preparations (Fig. 2, A and B). The substrate specificity of the transporter in the MXR-M ATPase assay correlated well with the substrate specificity in the vesicular transport inhibition experiment (Fig. 1F) and is in good agreement with published data (Litman et al., 2000; van der Heijden et al., 2004). The different behavior of the Sf9 membrane preparation could be a result of the dissimilarity in the membrane composition of insect and human membranes and/or in the glycosylation pattern of the transporters. Further studies are needed to gain insights into the molecular details behind the differences seen in the ATPase activities of the two membrane preparations.

The vanadate-sensitive ATPase activity of MXR-Sf9 membranes in the presence of Ko143 or Ko134 and the vanadate-sensitive ATPase activity present of membranes containing the defective transporter (MXR-K86M-Sf9-CTRL) show that the Sf9 preparations contain some (\( \sim 10 \) nmol Pi/mg/min) non–ABCG2-related vanadate-sensitive...
ATPase activity. This non–ABCG2-related activity was not present in MXR-M membranes (Fig. 2, C–F). Therefore, the MXR-M membrane is more suitable for the measurement of ABCG2 ATPase activity. When the basal ABCG2-dependent ATPase activity was suppressed by Ko143/Ko134, all the known substrates of ABCG2 stimulated the ATPase activity in both types of ABCG2 preparations (MXR-Sf9 and MXR-M). This shows that the reason for the unresponsiveness of the MXR-Sf9 preparation without inhibitors is not caused by altered substrate specificity of the transporter but is caused by the high baseline ATPase activity that cannot be further stimulated by ABCG2 substrates. By using ABCG2 inhibitors, the MXR-Sf9 membrane preparation is suitable for substrate identification, and it increases the signal-to-background ratio of the MXR-M preparation (Table 1). Because of the competitive nature of the interaction for sulfsalazine and Ko143/Ko134, using higher inhibitor concentrations results in an increased apparent EC$_{50}$ (Fig. 3, A–D). Therefore, the concentration of the inhibitor has to be set as a compromise between reasonable shift in EC$_{50}$ and adequately low baseline activities. For Ko143, this falls in the 50 to 100 nM range, whereas because of its somewhat lower affinity for ABCG2, it is in the 100 to 200 nM range for Ko134. For prazosin and topotecan, increasing Ko143/Ko134 concentrations does not result in an increased apparent EC$_{50}$, suggesting that these interactions are not competitive (Fig. 3, I and J). This suggests that Ko143/Ko134 binds to the same binding site as the hypothesized endogenous substrate, sulfsalazine and estrone-3-sulfate, whereas prazosin and topotecan bind to a different one. Multiple substrate/inhibitor binding sites for wild-type ABCG2 have been suggested previously (Ozvegy-Laczka et al., 2005). Our results also indicate the presence of more than one substrate binding site for the ABCG2 transporter.

Using the MXR-M membrane vesicles we measured different kinetics for methotrexate transport in the vesicular transport assay at pH 5.5 and 7.0. This is in accordance with vesicular transport studies conducted earlier using Sf9 membrane vesicles (Breedveld et al., 2007). The difference in vesicular transport correlated with the difference observed in the ATPase assay (Fig. 5, A and B). At pH 5.5, we observed precipitation in the assay buffer at 3 and 1 mM concentrations in both assays. This could be the explanation for the more prominent saturation of the transport observed at high concentrations at pH 5.5. For estrone-3-sulfate we measured identical transport rates in the vesicular transport assay and identical stimulation of the ATPase activity at both pH values in all the concentrations tested (Fig. 5, C and D). Sulfsalazine, topotecan, and prazosin also showed identical stimulation of ABCG2 ATPase activity at both pH values tested (Table 2). Further studies with more compounds would be necessary to identify substrates where transport is dependent on pH and substrates where it is not. Also, further studies are necessary to see whether differences measured in the vesicular transport assay are always reflected in the ATPase assay.

Estrone-3-sulfate was one of the best activators of the ATPase assay, indicating high transport rate for this compound. The transport can also be detected in the vesicular transport assay, which allowed us to compare the biochemical characteristics of ATPase stimulation and vesicular transport. The $K_m$ value for ATPase activation (22 µM) and
K_m for vesicular transport (7.8 μM) were comparable. The activation and transport could be inhibited by Ko134 in a similar, competitive fashion (Fig. 6). These results show that for estrone-3-sulfate kinetic parameters determined in the ATPase assay are reflective of the kinetic parameters of the actual transport.

In this work we successfully improved the ABCG2 ATPase assay to identify substrates of the transporter. We showed that the human membrane preparation containing ABCG2 can distinguish between substrates and nonsubstrates. Suppressing the baseline vanadate-sensitive ATPase activity by ABCG2 inhibitors Ko143 (50–100 nM) and Ko134 (100–200 nM) allowed us to increase the signal-to-background ratio of the assay. In essence, using the human membrane preparation containing ABCG2 with Ko134 or Ko143 is a reliable, sensitive, and robust assay that could be the preferred choice in screening for substrates of the ABCG2. Hits of this high-throughput, inexpensive method can be further characterized in lower-throughput and significantly more expensive direct transport experiments using vesicular transport or Transwell studies. This way, the improved ATPase assay can be a valuable tool in drug development as part of a screening strategy. We showed the feasibility of this approach when we successfully identified two new ABCG2 substrates using the improved ATPase assay and later successfully detected the ATP-dependent transport of both compounds in the vesicular transport assay.

The universal use of the ATPase assay to identify all the substrates of ABCG2 could be hampered by two obstacles. 1) It is well known that several compounds that were shown to be transported by ABCB1 (P-gp) do not stimulate the vanadate-sensitive ATPase activity of membrane preparations containing ABCB1. It is hypothesized that these compounds are transported by ABCB1 with a low turnover rate that does not yield detectable amount of inorganic phosphate in the ATPase assay. Our modified ABCG2 ATPase assay may also miss slowly transported compounds for similar reasons. 2) Previous work and our results suggest that the ABCG2 transporter has more than one binding site, resulting in a complex interaction profile for the different substrates and inhibitors of ABCG2. Therefore, the assay using Ko143 or Ko134 to inhibit the basal vanadate-sensitive ATPase activity creates a complex situation by the different kinetic interactions with drugs likely binding to different sites (Fig. 3; Table 1; discussion above). Additional studies using inhibitors binding to different sites to further improve the efficiency of the ATPase assay are warranted.

Acknowledgments. We thank Balázs Sarkadi (National Medical Center, Budapest, Hungary) and András Váradi (Inst Enzymology, Hungarian Academy of Sciences, Budapest, Hungary) for review of the manuscript.

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