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ABCG2 (BCRP/MXR) ATPase assay – a useful tool to detect drug – transporter interactions

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Abbreviations: MXR, mitoxantrone resistance-associated protein; BCRP, breast cancer resistance protein; ABCP, placenta specific ABC transporter; Pgp, P-glycoprotein; MRP, multidrug resistance-associated protein; Sf9 cells, *Spodoptera frugiperda* ovarian cells; H-33342, Hoechst 33342, MTX, methotrexate; E3S, estrone-3-sulfate

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Abstract

The ATPase assay utilizing membrane preparations from recombinant baculovirus infected Sf9 cells is widely used to detect the interaction of compounds with different ABC 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 i) we purified membranes from a selected human cell line expressing wild type ABCG2, and ii) 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.

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Introduction

ABCG2 (BCRP, MXR, ABC-P) 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 ATP binding cassette (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 (Kruijtz 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 (Pgp) (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 utilizing Sf9 membrane preparations is a widely used assay to test the interaction of compounds with different transporters including ABCB1/Pgp/MDR1 and members of the ABCC/MRP 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, might also indicate the nature of the interaction. Other available tools have the disadvantage of lower throughput and higher cost (transwell studies, direct vesicular transport), or provide less information on the nature of the interaction (inhibition studies).

The first ABCG2 ATPase assays utilizing Sf9 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). Sf9 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 Sf9 cells could be responsible for this phenomenon (Ozvegy et al., 2001).

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Membranes prepared from human expression systems have also been widely used in the field of ABC transporters (Sharom et al., 1999); (Loe et al., 1996); (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 (Rocchi et al., 2000); (Litman et al., 2000); (Robey et al., 2001); (Volk et al., 2000); (Volk et al., 2002). According to our hypothesis, membranes prepared from these cell lines might be suitable for ATPase assay. Furthermore, the different glycosylation pattern and/or membrane composition might 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 (Xia et al., 2005); (Allen et al., 2002), (Ozvegy et al., 2002); (Ozvegy-Laczka et al., 2004). 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 better signal-to-background ratios.

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Materials and Methods

Chemicals

[³H]-Methotrexate was purchased from Moravек Biochemicals (Brea, CA, USA). [³H]-estrone-3-sulfate was purchased from Perkin Elmer/NEN (Boston, MA, USA). Topotecan was purchased from LKT Laboratories (St. Paul, MN, USA). GF120918 was synthesized at Altana Pharma, Konstanz, Germany. BXP-21 antibody was purchased from Abcam (Cambridge, UK). All other compounds were purchased from Sigma.

Cell culture and membrane preparation

All ABCG2 preparations contained the wild type (482R) version of the ABCG2 transporter (acc. no. 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-Sf9) and control insect membranes (beta-gal-Sf9-CTRL, MXR-K86M-Sf9-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 (carring a mutation at a crucial position of the catalytic center of ATP binding and cleavage) and β -galactosidase (Ozvegy et al., 2001); (Ozvegy et al., 2002). Sf9 cells were cultured and infected with recombinant baculovirus stocks as described earlier (Sarkadi et al., 1992). Purified membrane vesicles from virus-infected Sf9 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-PAGE and subsequent western blotting using specific anti-ABCG2 antibody BXP-21, HRP-conjugated anti-mouse secondary antibody (Sigma) and enhanced chemiluminescence (ECL, Amersham Biosciences) as described earlier (Ozvegy et al., 2002).

ATPase assay

ATPase activity was measured as described earlier (Sarkadi et al., 1992). Briefly, membrane vesicles (20 μ g/well) were incubated in 10 mM MgCl₂, 40 mM MOPS-Tris (pH 7.0), 50 mM KCl, 5 mM

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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 as 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 were 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), 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 2x5 ml of ice cold wash buffer. When labeled compounds were used as substrates and radioactivity retained on the filter was measured by liquid scintillation counting. For unlabeled compounds filters were processed as described in the “Analytics” section. ATP-dependent transport was calculated by subtracting the values obtained in the presence of AMP from those in the presence of ATP.

Analytics

Estradiol-3,17-disulfate

Samples were subjected to sort isocratic HPLC separation before MS analysis. HPLC apparatus: Applied Biosystems 140C, the mobile phases were: methanol:water in a volume ratio 50:50%, flow rate: 250 μl/min; sample volume: 20 μl; column: Hypersil, 5 ODS, 20*2 mm. The samples were diluted in double distilled water.

Mass spectrometric (MS) measurements were obtained on a Finnigan TSQ-7000 triple quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with a Finnigan Electrospray Ionization (ESI) source. The instrument was operated in negative ion mode using selective reaction monitoring (SRM). In SRM mode the first quadrupole was set to select 215 *m/z* ((M-2H⁺)²⁻ of estradiol-3,17-disulfate) which was fragmented in the collision

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cell and the most intense fragment at 175 m/z (the double charged fragment ion showed a loss of HSO_3) was measured with the last quadrupole.

The ESI needle was adjusted to 4.5 kV and N_2 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 LC/DAD system (Agilent Technologies International sarl, Morges, Switzerland) was used, which consisted of the following modules: vacuum degasser (G1379A), 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.SR2. The equipment is coupled with a single quadrupole mass selective detector (MSD) (Agilent Technology, Wilmington, USA) 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 x 4 mm, particle size 2 μm , pore size 80A; Phenomenex, Torrance, USA) coupled with a Security Guard Fusion-RP C18 precolumn (4 mm x 3 mm I.D.; Phenomenex, Torrance, USA). 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, cone voltage 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 (SIM) at 294 m/z ($[\text{M}-\text{H}]^-$).

Statistical methods

Assays were run in duplicates and unbiased (“n-1”) standard deviation was calculated.

Cutoff values in screening were defined as:

$$\text{Cutoff} = \text{Baseline} + 3 * \text{Me},$$

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where Me is the median of the unbiased (“n-1”) standard deviations of the duplicate measurements of the dataset.

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Results

Correlations of ABCG2 activity in human (MXR-M) and in 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 (figure 1.A). 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 weight of 55-60 kDa. No band was detected in membrane preparations prepared from Sf9 cells infected with the baculovirus containing β -galactosidase gene (beta-gal-Sf9-CTRL, lane 3.). Membrane preparations from the selected human cell line exhibited a strong band with an apparent molecular weight 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. (figures 1.B-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 [3 H]-methotrexate vesicular transport measurements (figures 1.D-E.) revealed that [3 H]-methotrexate transport has similar K_M -values (3.9 mM and 3.6 mM for MXR-Sf9 and MXR-M membranes, respectively). Transport could be inhibited by the specific ABCG2 inhibitor Ko143 (1 μ M) in both preparations. Furthermore, the same substrate specificity was determined in the vesicular transport assay using various ABCG2 substrates (100 μ M) as an inhibition of methotrexate transport (figure 1.F.). The transport of 3 H-methotrexate could not be observed in Sf9 membranes containing the K86M defective mutant version of ABCG2, nor in membranes prepared from the unselected, ABCG2 non-overexpressing human cell lines (data not shown).

ABCG2 ATPase activity in MXR-M and in MXR-Sf9 membranes – effect of substrates and inhibitors

ABCG2 substrates sulfasalazine, topotecan and prazosine (Litman et al., 2000); (van der Heijden et al., 2004) showed only a slight modulation of the baseline vanadate sensitive ATPase activity of MXR-Sf9 membranes. Sulfasalazine appeared to be a weak stimulator, while topotecan and prazosine were weak inhibitors of the baseline activity (figure 2.A.). The ABCG2 in human cell membranes could be stimulated by all three compounds (figure 2.B.). All four inhibitors tested (Ko143, Ko134,

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Hoechst 33342 and GF120918) inhibited the baseline vanadate sensitive ATPase activity of the MXR-Sf9 preparation. Ko143, Ko134 and Hoechst 33342 inhibited the baseline vanadate sensitive ATPase activity of the MXR-M preparation. In contrast, GF120918 showed inhibition at lower concentrations, while it showed increasing ATPase activity above 1 μ M (figures 2.C-D). The MXR-Sf9 membrane preparation shows ~10 nmol Pi/mg/min 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, which was not modulated by any of the substrates tested (figure 2.E). The MXR-M membrane preparation shows no vanadate sensitive ATPase activity in the presence of 100 nM Ko143 or 200 nM Ko134. Control membranes prepared from the unselected, ABCG2 non-overexpressing human cell line also did not have any vanadate sensitive ATPase activity and could not be stimulated by any of the substrates tested (figure 2.F).

Drug-stimulated ABCG2 ATPase activity in a low background system

We have studied whether by suppressing the basal ATPase activity employing an ABCG2 inhibitor we can acquire a more sensitive system that allows the detection of substrate stimulated ATPase. Using different concentrations of sulfasalazine as activator we tested all four inhibitors at different concentrations on both membrane preparations. The inhibition of ABCG2 ATPase activity by Ko143 (figures 3.A-B.) and Ko134 (figures 3.C-D.) could be reversed by using increased concentrations of sulfasalazine. The apparent EC_{50} for the activating effect of sulfasalazine shifted to higher concentrations (for MXR-M membranes 1.5 μ M and 9.6 μ M in the presence of 0 and 80 nM Ko143, respectively) suggesting that these interactions are competitive. Due to 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 EC_{50} values than in the case of Ko143/Ko134 (figures 3.G-H.). Inhibition by Hoechst 33342 could not be reversed even by the highest concentration of sulfasalazine used (figures 3.E-F.). Therefore, inhibition by Ko143/Ko134 and GF120918 appeared to be competitive, while 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

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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 EC_{50} values suggesting that the interaction of these two compounds with Ko134 is not competitive (figure 3.I-J)

We screened a library of 30 compounds at 100 μ M using both membrane preparations with and without 100 nM Ko143 (table 1.). No interaction was detected with MXR-Sf9, yet using Ko143 allowed the detection of the known BCRP substrates sulfasalazine, topotecan and prazosine. The MXR-M preparation also detected the 3 known substrates in the absence of inhibitors, while applying Ko143 increased the signal-to-background (fold activation) values up-to more than 4-fold. No false positives 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-Sf9 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 (figure 4.).

The 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 [3 H]-methotrexate at pH 5.5 and pH 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 methotrexate, while 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,

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topotecan and prazosine also displayed similar stimulation of the ABCG2 ATPase activity when using the MXR-M preparation at pH 5.5 or pH 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 μM . It also showed a competitive interaction with Ko134 similar to that of sulfasalazine (figure 6.A-B.). In the vesicular transport assay estrone-3-sulfate was transported with a K_M of 7.8 μM and Ko134 inhibition was also competitive (figure 6.C-D.).

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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 as well as for [³H]-methotrexate. The MXR-M preparations showed significantly higher v_{\max} for [³H]-methotrexate transport (figure 1D, 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, while non-interacting drugs did not modulate the ATP dependent [³H]methotrexate transport in either membrane preparation (figure 1.F). 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, while stimulation was detected for all substrates tested for MXR-M preparations (figure 2.A-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 (figures 1.F), 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 granted 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 (~10 nmol Pi/mg/min) non-ABCG2 related vanadate sensitive ATPase activity. This non-ABCG2 related activity was not present in MXR-M membranes (figure 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 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 due to 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

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increases the signal-to-background ratio of the MXR-M preparation (table 1.). Due to the competitive nature of the interaction for sulfasalazine and Ko143/Ko134 using higher inhibitor concentrations results in an increased apparent EC_{50} .(figures 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 50-100 nM range, while due to its somewhat lower affinity for ABCG2 it is in the 100-200 nM range for Ko134. For prazosin and topotecan increasing Ko143/Ko134 concentrations do not result in an increased apparent EC_{50} suggesting that these interactions are not competitive (figure 3.I-J). This suggests that Ko143/Ko134 binds to the same binding site as the hypothesized endogenous substrate, sulfasalazine and estrone-3 sulfate, while prazosine and topotecan binds to a different one. Multiple substrate/inhibitor binding sites for wt 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 pH 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 (figure 5.A-B). At pH 5.5 we observed precipitation in the assay buffer at 3 mM 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 concentrations tested (figure 5.C-D). Sulfasalazine, topotecan and prazosine 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 activator 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 μ M) and K_M for vesicular transport (7.8 μ M) were comparable. The activation and transport could be inhibited by Ko134 in a similar, competitive fashion (figure 6.).

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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 non-substrates. Suppressing the baseline vanadate sensitive ATPase activity by ABCG2 inhibitors Ko143 (50-100 nM) or Ko134 (100-200nM) 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 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 (Pgp) 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 do not yield detectable amount of inorganic phosphate in the ATPase assay. Our modified ABCG2 ATPase assay might also miss slowly transported compounds for similar reasons. (2) Previous work and our results suggest that the ABCG2 transporter has more than one binding sites resulting in a complex interaction profile for the different substrates and inhibitors of ABCG2. Therefore, the assay utilizing 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., Table1., and discussion above). Additional studies utilizing inhibitors binding to different sites to further improve the efficiency of the ATPase assay are warranted.

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References

- Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, Schellens JH, Koomen GJ and Schinkel AH (2002) Potent and specific inhibition of the 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.
- Bakos E, Evers R, Sinko E, Varadi A, Borst P and Sarkadi B (2000) Interactions of the human multidrug resistance proteins MRP1 and MRP2 with organic anions. *Mol Pharmacol* **57**:760-768.
- Bensadoun A and Weinstein D (1976) Assay of proteins in the presence of interfering materials. *Anal Biochem* **70**:241-250.
- Bodo A, Bakos E, Szeri F, Varadi A and Sarkadi B (2003) Differential modulation of the human liver conjugate transporters MRP2 and MRP3 by bile acids and organic anions. *J Biol Chem* **278**:23529-23537.
- Breedveld P, Pluim D, Cipriani G, Dahlhaus F, van Eijndhoven MA, de Wolf CJ, Kuil A, Beijnen JH, Scheffer GL, Jansen G, Borst P and Schellens JH (2007) The effect of low pH on breast cancer resistance protein (ABCG2)-mediated transport of methotrexate, 7-hydroxymethotrexate, methotrexate diglutamate, folic acid, mitoxantrone, topotecan, and resveratrol in in vitro drug transport models. *Mol Pharmacol* **71**:240-249.
- Dietrich CG, Geier A and Oude Elferink RP (2003) ABC of oral bioavailability: transporters as gatekeepers in the gut. *Gut* **52**:1788-1795.
- Hirohashi T, Suzuki H, Chu XY, Tamai I, Tsuji A and Sugiyama Y (2000) Function and expression of multidrug resistance-associated protein family in human colon adenocarcinoma cells (Caco-2). *J Pharmacol Exp Ther* **292**:265-270.
- Honjo Y, Hrycyna CA, Yan QW, Medina-Perez WY, Robey RW, van de Laar A, Litman T, Dean M and Bates SE (2001) Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells. *Cancer Res* **61**:6635-6639.
- Kruijtzter CM, Beijnen JH, Rosing H, ten Bokkel Huinink WW, Schot M, Jewell RC, Paul EM and Schellens JH (2002) Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. *J Clin Oncol* **20**:2943-2950.
- Litman T, Brangi M, Hudson E, Fetsch P, Abati A, Ross DD, Miyake K, Resau JH and Bates SE (2000) The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). *J Cell Sci* **113** (Pt 11):2011-2021.
- Litman T, Druley TE, Stein WD and Bates SE (2001) From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell Mol Life Sci* **58**:931-959.
- Loe DW, Almquist KC, Deeley RG and Cole SP (1996) Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport. *J Biol Chem* **271**:9675-9682.
- Ozvegy C, Litman T, Szakacs G, Nagy Z, Bates S, Varadi A and Sarkadi B (2001) Functional characterization of the human multidrug transporter, ABCG2, expressed in insect cells. *Biochem Biophys Res Commun* **285**:111-117.

DMD 14605

- Ozvegy C, Varadi A and Sarkadi B (2002) Characterization of drug transport, ATP hydrolysis, and nucleotide trapping by the human ABCG2 multidrug transporter. Modulation of substrate specificity by a point mutation. *J Biol Chem* **277**:47980-47990.
- Ozvegy-Laczka C, Hegedus T, Varady G, Ujhelly O, Schuetz JD, Varadi A, Keri G, Orfi L, Nemet K and Sarkadi B (2004) High-affinity interaction of tyrosine kinase inhibitors with the ABCG2 multidrug transporter. *Mol Pharmacol* **65**:1485-1495.
- Ozvegy-Laczka C, Koblos G, Sarkadi B and Varadi A (2005) Single amino acid (482) variants of the ABCG2 multidrug transporter: major differences in transport capacity and substrate recognition. *Biochim Biophys Acta* **1668**:53-63.
- Robey RW, Medina-Perez WY, Nishiyama K, Lahusen T, Miyake K, Litman T, Senderowicz AM, Ross DD and Bates SE (2001) Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. *Clin Cancer Res* **7**:145-152.
- Rocchi E, Khodjakov A, Volk EL, Yang CH, Litman T, Bates SE and Schneider E (2000) The product of the ABC half-transporter gene ABCG2 (BCRP/MXR/ABCP) is expressed in the plasma membrane. *Biochem Biophys Res Commun* **271**:42-46.
- Sarkadi B, Ozvegy-Laczka C, Nemet K and Varadi A (2004) ABCG2 -- a transporter for all seasons. *FEBS Lett* **567**:116-120.
- Sarkadi B, Price EM, Boucher RC, Germann UA and Scarborough GA (1992) Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. *J Biol Chem* **267**:4854-4858.
- Sharom FJ, Yu X, Lu P, Liu R, Chu JW, Szabo K, Muller M, Hose CD, Monks A, Varadi A, Seprodi J and Sarkadi B (1999) Interaction of the P-glycoprotein multidrug transporter (MDR1) with high affinity peptide chemosensitizers in isolated membranes, reconstituted systems, and intact cells. *Biochem Pharmacol* **58**:571-586.
- van der Heijden J, de Jong MC, Dijkmans BA, Lems WF, Oerlemans R, Kathmann I, Scheffer GL, Scheper RJ, Assaraf YG and Jansen G (2004) Acquired resistance of human T cells to sulfasalazine: stability of the resistant phenotype and sensitivity to non-related DMARDs. *Ann Rheum Dis* **63**:131-137.
- van Herwaarden AE and Schinkel AH (2006) The function of breast cancer resistance protein in epithelial barriers, stem cells and milk secretion of drugs and xenotoxins. *Trends Pharmacol Sci* **27**:10-16.
- Volk EL, Farley KM, Wu Y, Li F, Robey RW and Schneider E (2002) Overexpression of wild-type breast cancer resistance protein mediates methotrexate resistance. *Cancer Res* **62**:5035-5040.
- Volk EL, Rohde K, Rhee M, McGuire JJ, Doyle LA, Ross DD and Schneider E (2000) Methotrexate cross-resistance in a mitoxantrone-selected multidrug-resistant MCF7 breast cancer cell line is attributable to enhanced energy-dependent drug efflux. *Cancer Res* **60**:3514-3521.
- Xia CQ, Liu N, Yang D, Miwa G and Gan LS (2005) Expression, localization, and functional characteristics of breast cancer resistance protein in Caco-2 cells. *Drug Metab Dispos* **33**:637-643.

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Footnotes

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Conflict of interest statement

None of the authors has any financial or personal relationships to disclose that could potentially be perceived as influencing the described research.

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Legends for figures

Figure 1:

(A) ABCG2 levels in the different membrane preparations as detected by the monoclonal antibody BXP-21 in western blot. 10 μ g of total membrane protein/sample were separated by 10% SDS-PAGE and blotted on PVDF membranes. The presence of ABCG2 was detected by the monoclonal antibody BXP-21 and anti-mouse-HRP secondary antibody visualized with ECL 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 μ g 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 minutes and 4 minutes for MXR-Sf9 membrane vesicles and MXR-M vesicles, respectively. Insert: Lineweaver-Burk plot. Inhibition of the ATP dependent [³H]-methotrexate transport (100 μ M) for Sf9 and human membrane vesicles by different substrates and non-substrates (100 μ M) and the specific inhibitor Ko143 (1 μ M) of ABCG2 (F).

Figure 2:

Vanadate sensitive ATPase activity of MXR-Sf9 (A, C) and MXR-M (B, D), defMXR-K86M-Sf9 (E) and M-CTRL (F) preparations in the presence of ABCG2 substrates and inhibitors at different concentrations. Membranes containing 20 μ g of total protein were incubated at 37 °C for 40 min in the presence of different concentrations of test compounds.

Figure 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 (legend, A-B Ko143 (nM), C-D Ko134 (nM), E-F Hoechst 33342 (μ M), G-H GF120918 (μ M)). Vanadate sensitive ATPase activity plotted as relative activity (%) of the MXR-M membrane preparation in the presence of different concentrations of prazosine (I) or topotecan (J) and Ko134 (legend, nM). Membranes containing 20 μ g of total protein were incubated at 37 °C for 40 min.

Figure 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

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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.

Figure 5:

Vanadate sensitive ATPase activity plotted as relative activity (%) of MXR-M membrane preparations incubated at the indicated pH in the presence of different concentrations of methotrexate (A) or estrone-3-sulfate (C). Membranes containing 20 μ g of total protein were incubated at 37 °C for 40 min. ATP dependent transport of [³H]-methotrexate (B) or [³H]-estrone-3-sulfate (D) at the indicated pH at different substrate concentrations. Incubation time was 4 minutes and 1 minute, respectively.

Figure 6:

(A) Vanadate sensitive ATPase activity of MXR-M preparations in the presence of different concentrations of estrone-3-sulfate and Ko134 (nM, legend). Membranes containing 20 μ g of total protein were incubated at 37 °C for 40 min. (B) Lineweaver-Burk plot of the estrone-3-sulfate stimulated vanadate sensitive ATPase activity ($v-v(0)$) of MXR-M preparations in the presence of different concentrations of Ko134 (nM, legend). $v-v(0)$ was calculated by subtracting the vanadate sensitive ATPase activity in the presence of the respective Ko134 concentration from the vanadate sensitive ATPase activity determined in the presence of both estrone-3-sulfate Ko134. (C) ATP dependent [³H]-estrone-3-sulfate transport for MXR-M membrane vesicles at different estrone-3-sulfate concentrations in the presence of different concentrations of Ko134 (nM, legend). Incubation time was 1 minute. (D) Lineweaver-Burk plot of figure C.

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Tables

Table 1. Fold activation (signal-to-background) values measured for the different assays in the presence of the compounds listed (100 μ M). Values greater than the respective cutoff values are shaded.

	Assay system (cutoff value)			
	MXR- Sf9 (1.22)	MXR- Sf9 + Ko143 (1.17)	MXR-M (1.23)	MXR-M + Ko143 (1.97)
Acyclovir	0.9	1.0	1.1	1.1
Alprenolol-HCl	0.9	1.0	1.1	0.6
Amilorid	1.0	0.9	1.1	1.1
Antipyrin	0.9	0.9	1.0	1.0
Atenolol	1.0	1.0	1.0	1.2
Ceftriaxon	0.9	1.0	0.9	1.1
Chloramphenicol	0.4	1.1	0.9	1.7
Desipramin	1.0	0.9	0.6	0.2
Digoxin	0.2	1.0	0.3	0.8
Doxorubicin	0.8	1.1	1.1	0.1
Erythromycin	0.9	1.1	0.9	0.7
Guanabenz	0.5	1.0	1.0	0.2
Imipramine	0.9	0.9	0.6	0.0
Lansoprazol	0.2	1.0	0.4	0.9
Levodopa	0.9	1.0	1.0	0.4
Metoprolol	1.0	1.0	1.0	0.6
Naproxen	0.5	1.1	1.0	0.6
Omeprazol	0.5	1.1	0.8	0.5
Prazosin	0.7	1.4	1.5	5.0
Propranolol	0.8	1.0	1.0	1.0
Pumafentrin	0.6	0.8	0.6	0.1
Quinidin	0.4	0.9	0.8	0.3
Ranitidine	1.0	1.0	1.2	0.8
Ritonavir	0.1	0.9	0.0	0.1
Sulfasalazine	1.0	2.4	2.0	7.2
Sulpirid	0.9	1.0	1.0	0.6
Terbutalin	0.5	1.0	0.9	0.7
Testosteron	0.7	1.1	0.4	0.0
Topotecan	0.8	1.3	2.1	2.7
Verapamil	0.4	1.0	0.7	0.2

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Table 2.

EC₅₀* and maximal fold activation (signal-to-background) values for stimulation of vanadated sensitive ATPase activity of MXR-M membranes at different pH values. Values presented are averages and standard deviations of two independent experiments.

	pH 5.5		pH 7.0	
	EC ₅₀ (μM)*	Maximal fold activation	EC ₅₀ (μM)*	Maximal fold activation
Sulfasalazine	0.6 ± 0.1	2.9 ± 0.6	0.6 ± 0.1	3.4 ± 1.0
Prazosin	4.5 ± 0.7	2.3 ± 0.6	2.5 ± 0.7	2.0 ± 0.6
Topotecan	25 ± 7.1	2.5 ± 0.2	17.5 ± 3.5	2.4 ± 1.1

* The concentration of half maximal stimulation of the compound tested

Figure 1

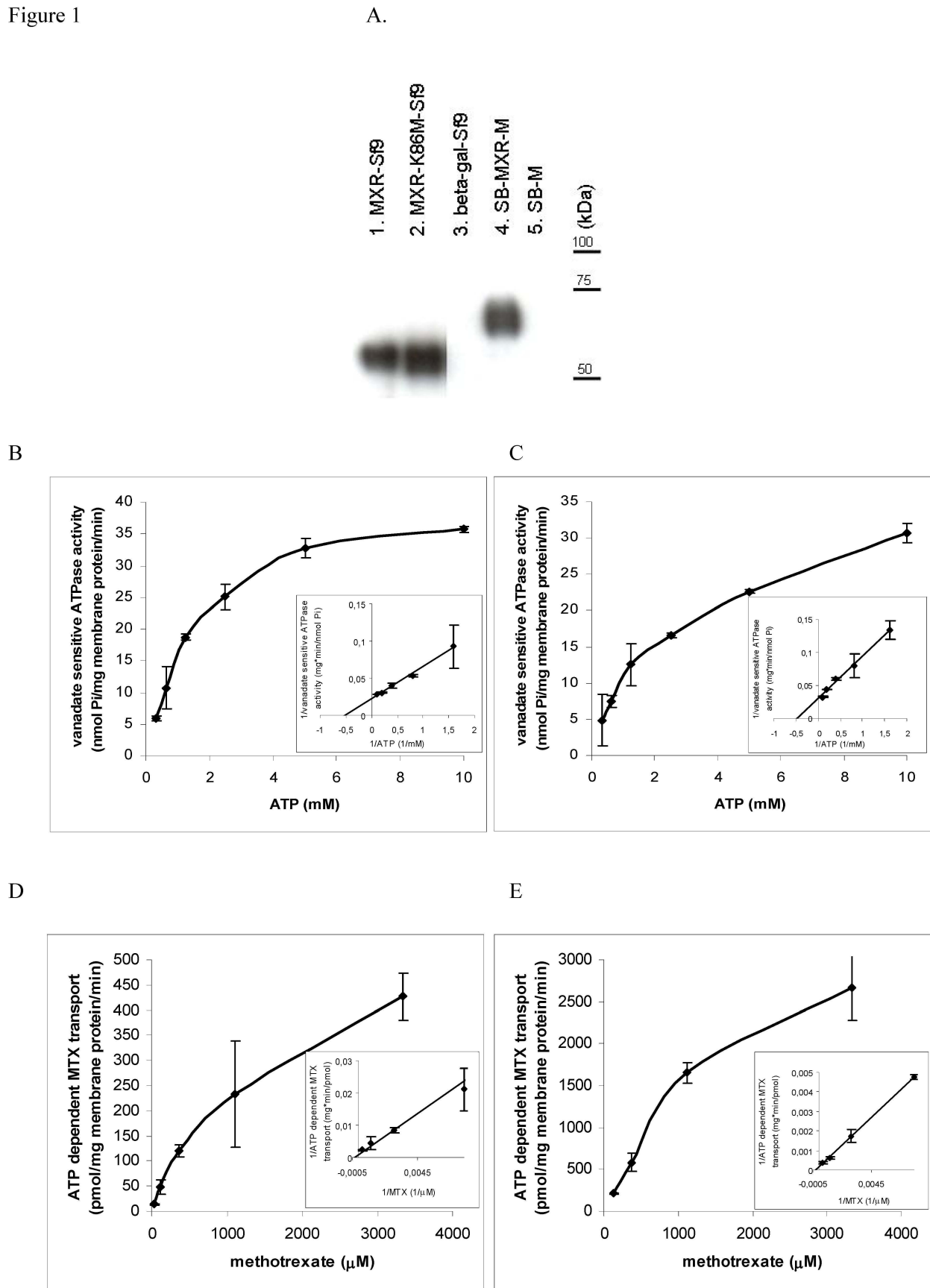


Figure 1 - continued

F.

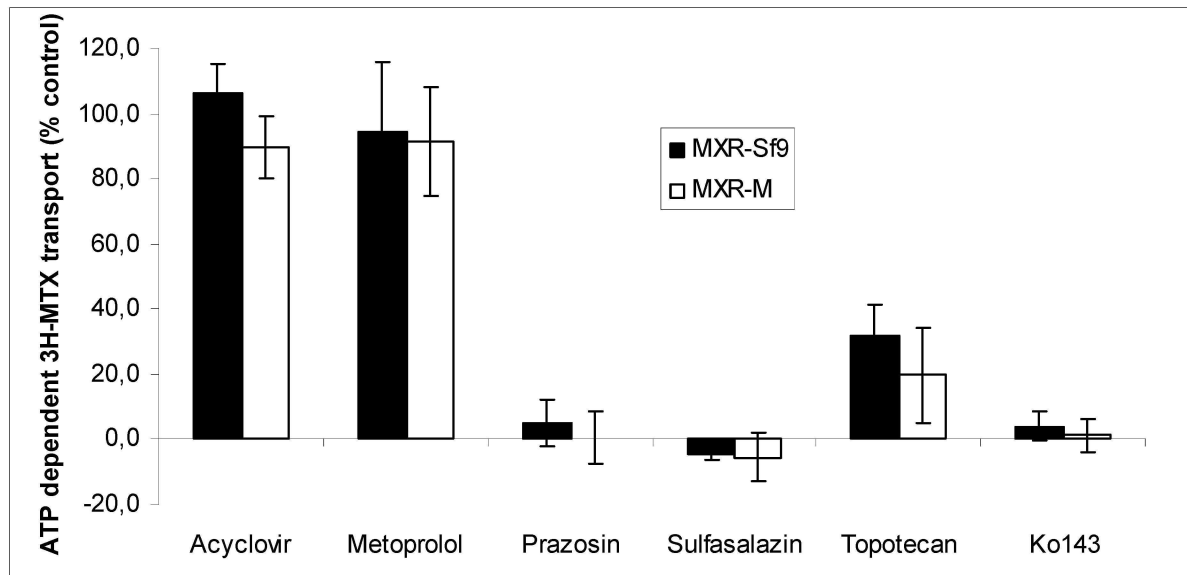
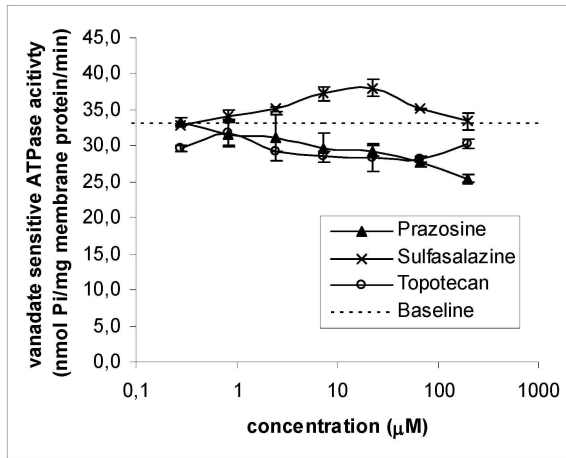
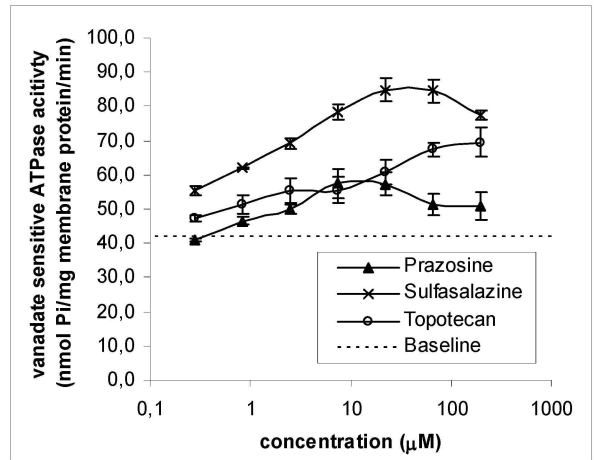


Figure 2

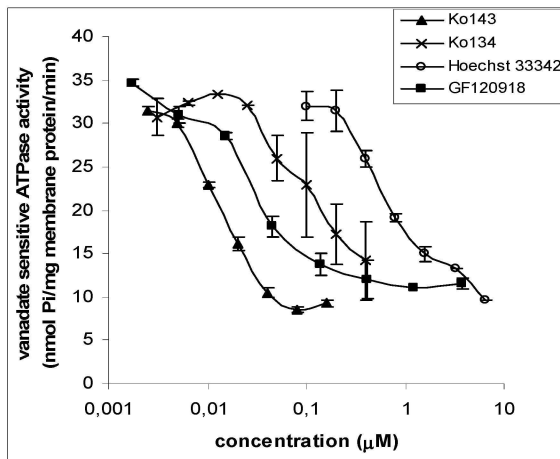
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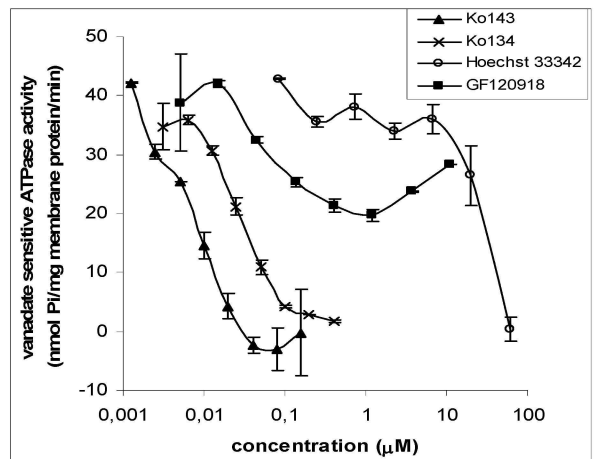
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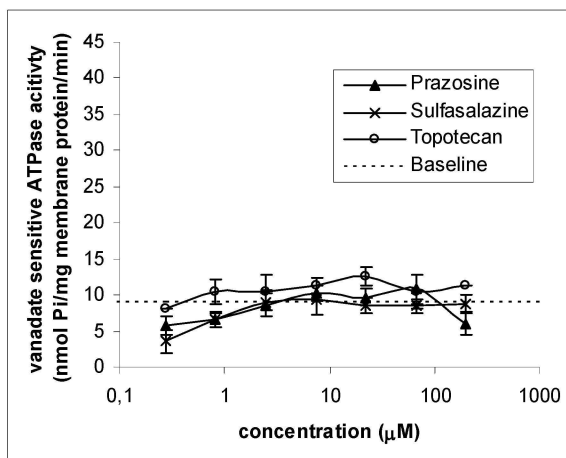
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D.



E.



F.

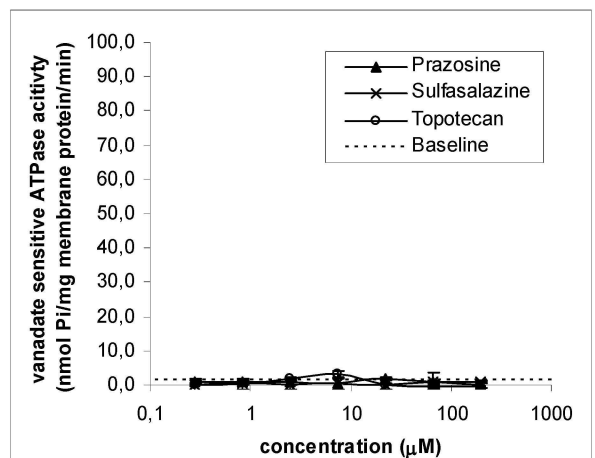


Figure 3

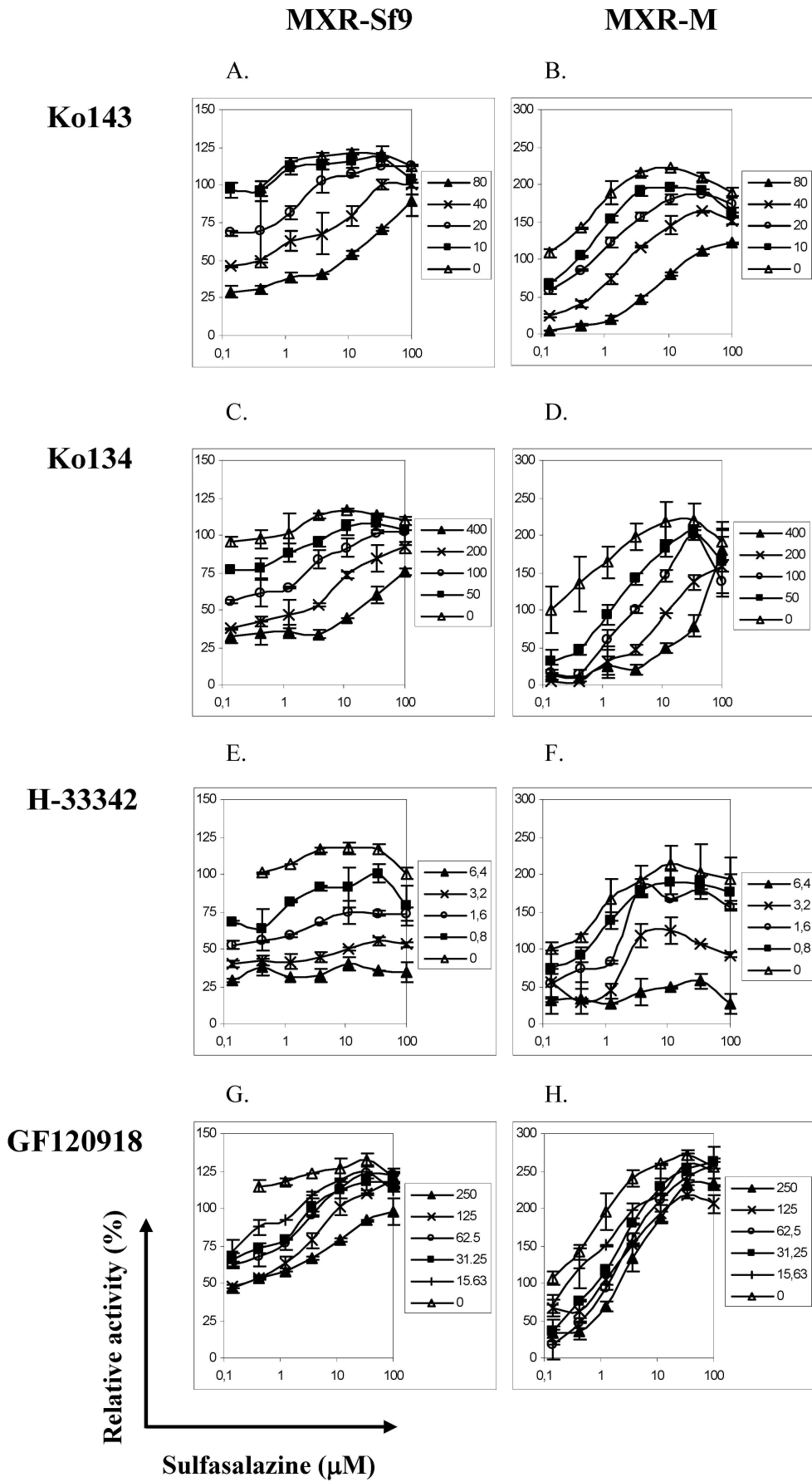
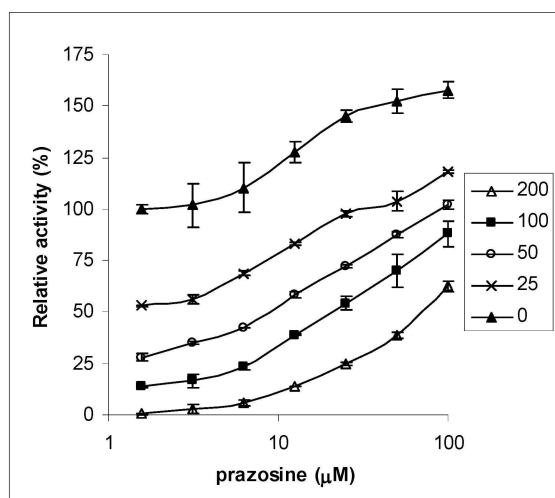


Figure 3 - continued

I.



J.

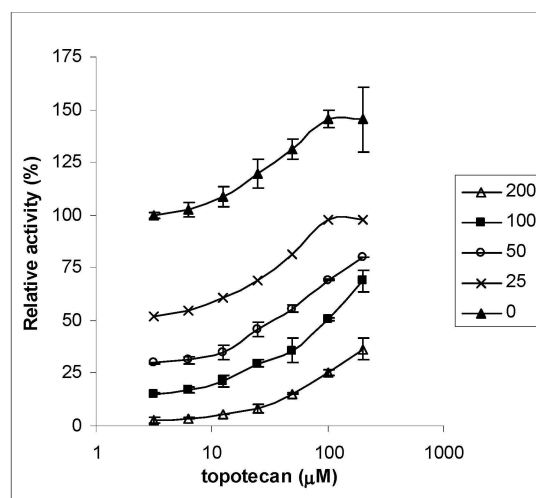
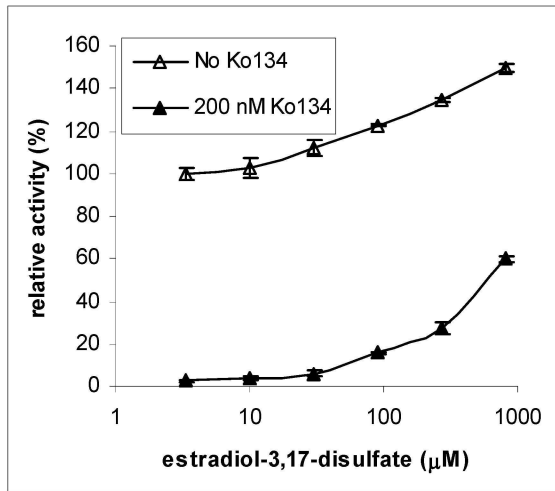
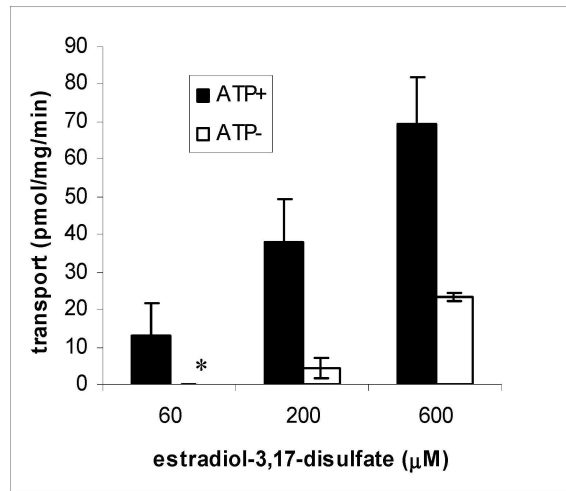


Figure 4.

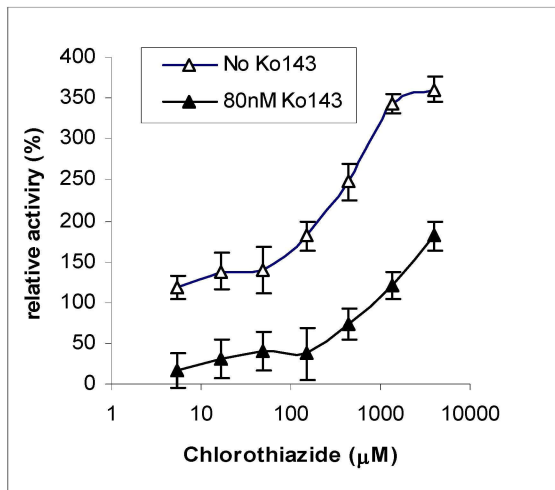
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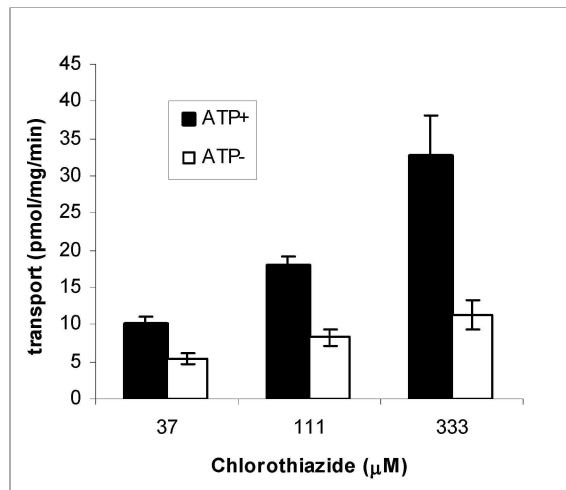
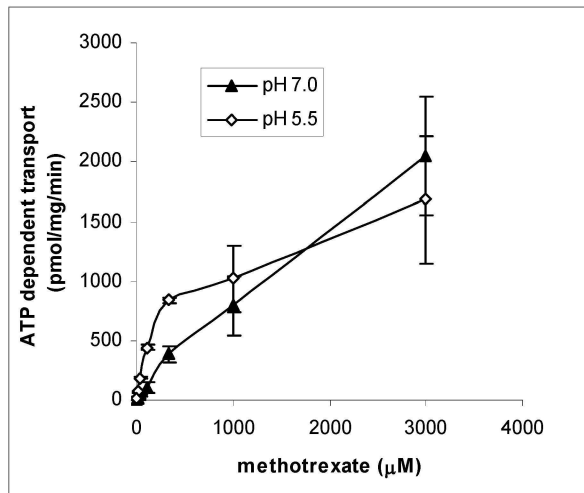
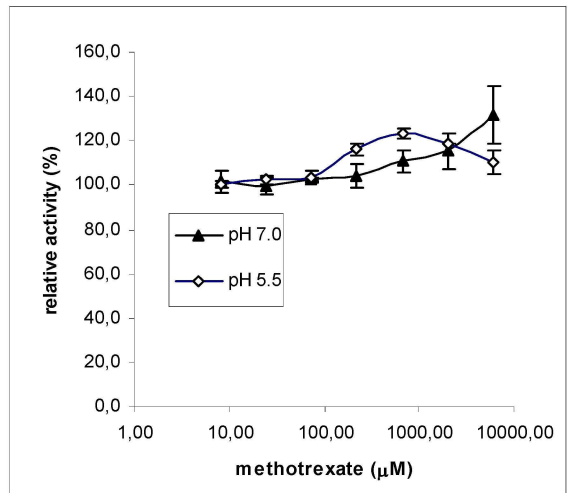


Figure 5

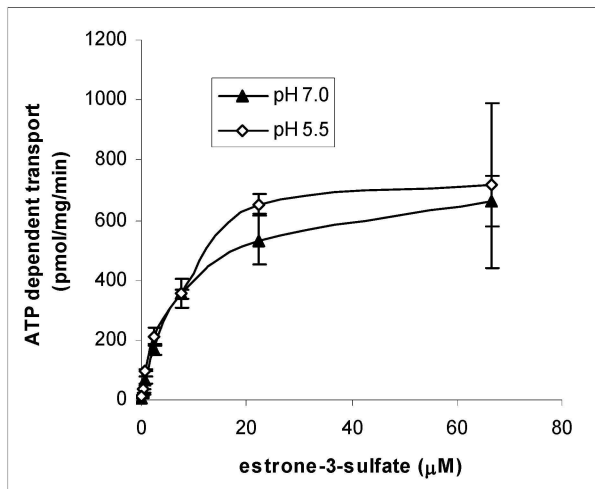
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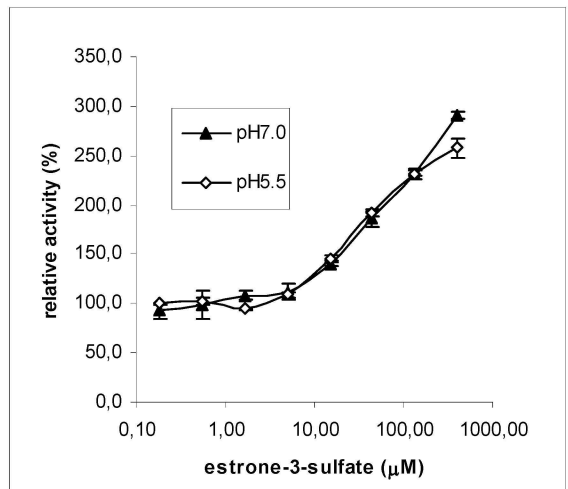


Figure 6.

