J Membrane Biol (2015) 248:967–977
DOI 10.1007/s00232-015-9804-y

ORIGINAL ARTICLES

Membrane Assays to Characterize Interaction of Drugs with ABCB1

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Received: 7 January 2015 / Accepted: 20 April 2015 / Published online: 30 April 2015

Abstract ATP-binding cassette sub-family B member 1 (ABCB1) [P-glycoprotein (P-gp), multidrug resistance protein 1 (MDR1)] can affect the pharmacokinetics, safety, and efficacy of drugs making it important to identify compounds that interact with ABCB1. The ATPase assay and vesicular transport (VT) assay are membrane based assays that can be used to measure the interaction of compounds with ABCB1 at a lower cost and higher throughput compared to cellular-based assays and therefore can be used earlier in the drug development process. To that end, we tested compounds previously identified as ABCB1 substrates and inhibitors for interaction with ABCB1 using the ATPase and VT assays. All compounds tested interacted with ABCB1 in both the ATPase and VT assays. All compounds previously identified as ABCB1 substrates activated ABCB1-mediated ATPase activity in the ATPase assay. All compounds previously identified as ABCB1 inhibitors inhibited the ABCB1-mediated transport in the VT assay. Interestingly, six of the ten compounds previously identified as ABCB1 inhibitors activated the basal ATPase activity in activation assays suggesting that the compounds are substrates of ABCB1 but can inhibit ABCB1 in inhibition assays. Importantly, for ATPase activators the EC_{50} of activation correlated with the IC_{50} values from the VT assay showing that interactions of compounds with ABCB1 can be measured with similar levels of potency in either assay. For ATPase nonactivators the IC_{50} values from the ATPase inhibition and VT inhibition assay showed correlation. These results demonstrate the utility of membrane assays as tools to detect and rank order drug–transporter interactions.

Keywords ADME · ATPase assay · MDR1 · Multidrug resistance transporters · P-gp · Vesicular transport assay

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Introduction

ATP-binding cassette sub-family B member 1 (ABCB1), also known as P-glycoprotein (P-gp) or multidrug resistance protein 1 (MDR1), has been identified as an important determinant of pharmacokinetic properties of drugs and as a mediator of drug–drug interactions by the International Transporter Consortium (ITC) (Giacomini et al. 2010), the Food and Drug Administration (FDA) (FDA-Guidance 2012) and the European Medicines Agency (EMA) (EMA-Guidance 2012). In vitro methods to identify ABC transporter substrates include assays with intact cells such as the monolayer efflux assay on transwell plates, cellular uptake, and cytotoxicity assays, as well as membrane-based assays that include the ATPase assay and the vesicular transport (VT) assay. Since cellular uptake, cytotoxicity, and VT assays function by retaining substrates of the transporter in the cell or vesicle, these assays do not work well with compounds that have an intermediate or high passive permeability. Since the permeability

of ABCB1 substrates may include compounds that range from intermediate to high passive permeability, the monolayer efflux assay on transwell plates has been considered the gold standard to identify ABCB1 substrates and inhibitors for drug development and regulatory purposes (EMA-Guidance 2012; FDA-Guidance 2012). The ATPase assay which monitors transporter-mediated ATP hydrolysis linked to drug transport (Sarkadi et al. 1992; Sauna et al. 2006; Xia et al. 2006) has been used for large scale screening, as the colorimetric read-out makes it an attractive option for high-throughput screening (Schwab et al. 2003). The VT inhibition assay has also been reported to be an acceptable alternative method for studying low passive permeability drugs (Zhang et al. 2009). In this study, we measured the ability of compounds previously identified as substrates or inhibitors of ABCB1 in cellular-based assays to interact with ABCB1 in the ATPase or VT assay.

The first systematic correlation between monolayer efflux on transwell plates and ATPase assays was carried out by Polli et al. (2001). In that study, the bidirectional permeability of compounds across MDCKII-MDR1 cells (Madin-Darby canine kidney strain II cells overexpressing ABCB1) was measured and compared to effect the compound had on the ATPase activity as measured in the ATPase assay with Sf9 (*Spodoptera frugiperda*) membranes containing ABCB1. Compounds showing transporter-dependent permeability across MDCKII-MDR1 cells (defined as having an efflux ratio (ER) ≥ 2) were designated as transported substrates. Compounds that activated the ATPase activity, but lacked transporter-dependent permeability across MDCKII-MDR1 cells (defined as having an ER < 2) were labeled as nontransported substrates. It is of note that high passive permeability compounds were overrepresented in the group identified as nontransported substrates. The observation that the monolayer efflux assay on transwell plates generates false negatives in the group of high passive permeability compounds was confirmed by von Richter et al. 2009 (von Richter et al. 2009).

During the past few years, a number of publications by regulatory agencies have reviewed the field (EMA-Guidance 2010, 2012; FDA-Guidance 2006, 2012; Giacomini et al. 2010) In this study, we tested compounds identified as inhibitors and substrates of ABCB1 in the 2006 FDA guidance on drug–drug interactions (FDA-Guidance 2006) with the ATPase activation assay, and correlated it with the classification (substrate vs inhibitor) previously indicated, as well as transport data available from the literature. In addition, we tested compounds as inhibitor of ABCB1 in the ATPase and VT inhibition assays to compare IC_{50} (half maximal inhibitory concentration) values generated using these assays, as well as a correlation between VT IC_{50} values and ATPase EC_{50} (half maximal effective concentration) data.

Materials and Methods

Materials

[^{3}H]-*N*-methyl-quinidine was obtained from Dr. Csaba Tömböly (Biological Research Center, Hungary).

Fetal bovine serum (Lonza, DE14-802F), Eagle's Minimum Essential Medium (EMEM, Lonza, 12-662-500ML), Dulbecco's Modified Eagle's Medium (DMEM, Lonza, 12-708F), penicillin–streptomycin (Lonza, 09-757F) were purchased from Biocenter Kft. (Szeged, Hungary).

L-Glutamine (G3126) and other chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Kft, Budapest, Hungary) and were of analytical grade.

Membranes

Membrane vesicle preparations from baculovirus-infected Sf9 cells expressing human ABCB1 (MDR1/P-gp-Sf9) and from mammalian cells overexpressing ABCB1 (MDR1/P-gp-K) were from SOLVO Biotechnology (Budaörs, Hungary). Membrane protein content was determined using the bicinchonic acid (BCA) method, according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL).

ATPase Activity

ABC transporters hydrolyze ATP to ADP as an energy source to actively transport compounds across cell membranes against a concentration gradient. Transport function can be inferred by monitoring the rate of ATP hydrolysis or ATPase activity. The ATPase activity of ABC transporters can be modulated by interacting compounds. Substrates of the transporter will activate the ATPase activity whereas inhibitors will reduce the ATPase activity. This ATPase activity was measured using ABC transporter-containing membrane preparations, as described previously (von Richter et al. 2009). The rate of ATP hydrolysis was determined by measuring the liberation of inorganic phosphate using PREDEASY ATPase kits for MDR1/P-gp, from (SOLVO Biotechnology, Budaörs, Hungary) according to the manufacturer's instructions.

In brief, to measure if compounds activate ATPase activity, membrane vesicles were incubated with various concentrations of test drugs. To measure if compounds inhibit ATPase activity, membranes were incubated with a known substrate of the transporter (40 μM verapamil) to fully activate the ATPase activity along with various concentrations of the test compound. Incubations were carried out in the presence or absence of 1.2 mM sodium orthovanadate, an inhibitor of ABC transporter ATPase activity, such that the vanadate-sensitive ATPase activity represents

the ABC transporter-specific activity. Results are presented as vanadate-sensitive ATPase activities. Assays were run in duplicates and performed at least twice. EC_{50} and IC_{50} values were determined as described below.

Vesicular Transport Assay

Inhibition of ABCB1 in the VT transport was performed with ABCB1-overexpressing mammalian membranes according to the manufacturer's suggestions. Briefly, [^{3}H]-*N*-methyl-quinidine, a low permeability substrate of ABCB1, was incubated with membrane fractions containing inside-out membrane vesicles. Incubations were carried out in a 96-well plate in the presence or absence of 4 mM ATP. [^{3}H]-*N*-methyl-quinidine accumulates in the ABCB1 expressing vesicles in the presence of ATP but not in the absence of ATP. Inhibitors of ABCB1 will block the accumulation of the [^{3}H]-*N*-methyl-quinidine in the vesicles. Reactions were stopped by the addition of ice-cold washing buffer, followed by rapid filtration through 1.0 µm pore size, class B glass fiber filters combined with 0.65-µm pore size Durapore (PVDF) membrane in a 96-well filter plate set-up (MSFBN6B10, Millipore Corporation, Billerica, MA). After washing five times with 200 µl ice-cold washing buffer the filters were dried and the radioactivity retained was measured following addition of scintillation cocktail (Packard UltimaGold, Perkin-Elmer, Waltham, MA, USA) using a Wallac MicroBeta TriLux (Perkin-Elmer, Waltham, MA, USA) liquid scintillation counter. ATP-dependent transport was calculated as the difference in probe substrate transport in the presence or absence of ATP. Inhibition experiments were carried out using a 3 min incubation at 37 °C for ABCB1 membranes during which time the assays were still in the linear range (Heredi-Szabo et al. 2013). Assays were run in duplicates and performed at least twice. IC_{50} values were determined as described below.

Data Analysis

ATPase Kinetics

Experimental data were analyzed using Prism 4.0 (GraphPad Software SanDiego, CA, USA). EC_{50} values were determined from a sigmoidal dose–response curve fitted onto the vanadate-sensitive ATPase activity versus concentration plot by non-linear regression, using the following equation:

$$Y = V_{\min} + \frac{(V_{\max} - V_{\min})}{1 + 10^{(\log \mathrm{EC50} - \log[A]) \times \mathrm{nH}}},$$

where Y is the vanadate-sensitive ATPase activity, $V_{\min}$ is the minimal activation observed, $V_{\max}$ is the maximal activation observed, $[A]$ is the concentration of the test drug, EC_{50} is the concentration corresponding to a response that is halfway between $V_{\min}$ and $V_{\max}$, and the Hill slope (nH) is the parameter characterizing the degree of cooperativity. The IC_{50} values were determined from a sigmoidal dose–response curve using the same equation and IC_{50} corresponds to EC_{50} in the equation.

Some drugs caused a stimulatory response at low concentrations, and an inhibitory response at high concentrations. Bell-shaped dose–response curves were fitted onto these data using the following equations:

$$\mathrm{Span1} = \mathrm{Plateau1} - \mathrm{Dip},$$

$$\mathrm{Span2} = \mathrm{Plateau2} - \mathrm{Dip},$$

$$\mathrm{Section1} = \frac{\mathrm{Span1}}{1 + 10^{(\mathrm{logEC501} - X) \times \mathrm{nH1}}},$$

$$\mathrm{Section2} = \frac{\mathrm{Span2}}{1 + 10^{(\mathrm{logEC502}) \times \mathrm{nH2}}},$$

$$Y = \mathrm{Dip} + \mathrm{Section1} + \mathrm{Section2},$$

where Plateau1 and Plateau2 are the plateaus at the left and right ends of the curve, dip is the plateau level in the middle of the curve, $\mathrm{LogEC_{50}}1$ and $\mathrm{LogEC_{50}}2$ are the concentrations that give half-maximal stimulatory and inhibitory effects, nH1 and nH2 are the unitless slope factors or Hill slopes.

Vesicular Transport

For data analysis, Prism 4.0 (GraphPad Software, San-Diego, CA, USA) was applied. The results of the VT inhibition assays were analyzed using the same dose response vs. inhibition equation (variable slope sigmoid equation) which was used for ATPase activity:

$$Y = V_{\min} + \frac{(V_{\max} - V_{\min})}{1 + 10^{(\log \mathrm{EC50} - \log[A]) \times \mathrm{nH}}},$$

where Y is velocity (picomoles of substrate per milligram of protein per minute), $V_{\min}$ is minimal velocity (fully inhibited transport), $V_{\max}$ is maximal velocity (in the absence of inhibitor), IC_{50} is the ligand concentration corresponding to a response that is halfway between $V_{\min}$ and $V_{\max}$, $[A]$ is the actual test drug concentration, and the Hill slope (nH) is the parameter characterizing the degree of cooperativity.

Results

ABCB1 ATPase Activation and Inhibition

Compounds previously identified as substrates of ABCB1 were tested as activators or inhibitors of ABCB1 ATPase

activity and inhibitors of ABCB1 in the VT assay. Compounds were tested across concentration ranges exceeding three orders of magnitude. Six out of seven compounds (digoxin, fexofenadine, indinavir, vincristine, colchicine, and paclitaxel) classified as ABCB1 substrates (FDA-Guidance 2006) activated the transporter ATPase at a wide concentration range with EC_{50} values ranging from 0.199 μM (paclitaxel) to 321 μM (colchicine) (Table 1; Figs. 1a–e, g, 2). Topotecan, also classified as a substrate displayed a statistically significant activation only at the highest concentration tested (Fig. 1f) and higher concentrations could not be used because of limited solubility of the drug. Therefore, an EC_{50} value for this interaction was not determined (Table 1). With the exception of fexofenadine, colchicine, and topotecan, all the compounds exhibited maximum type biphasic curves in the concentration range tested (Fig. 1).

Inhibition of ABCB1 by compounds classified as substrates was examined using ATPase inhibition and VT inhibition assays, with verapamil as an activator and *N*-methyl-quinidine (NMQ) as a probe substrate, respectively. All compounds except colchicine and topotecan inhibited the verapamil-induced ABCB1 ATPase activity (Fig. 1). Colchicine (Fig. 1e) exerted some inhibition at the highest concentration tested. All compounds in this group inhibited ABCB1-mediated NMQ transport (Figs. 1, 2). Topotecan-mediated inhibition of NMQ transport was partial (Fig. 1f), and was insufficient to derive an IC_{50} value. For compounds which activated ABCB1 ATPase there was an acceptable correlation between the VT IC_{50} values and ATPase EC_{50} values, with the notable exceptions of digoxin and fexofenadine (Table 1; Fig. 2). Also, the ranking of potencies for ATPase activators was similar (paclitaxel ∼ indinavir > vincristine > fexofenadine ∼ digoxin > colchicine ∼ topotecan) in ATPase activation and VT inhibition.

Compounds previously identified as inhibitors of ABCB1 were tested as activators or inhibitors of ABCB1 ATPase activity and inhibitors of ABCB1 in the VT assay. Interestingly, six of the ten compounds listed as ABCB1 inhibitors (ritonavir, erythromycin, quinidine, verapamil, ketoconazole, and itraconazole) (FDA-Guidance 2006) activated the ATPase activity of the transporter in the ATPase activation assay (Fig. 3a–f). Only compounds that were considered reversal agents (cyclosporin A, PSC833, GF120918, and LY335979) were inactive in this assay (Fig. 3g–j).

The six inhibitors with ATPase activator properties inhibited NMQ transport using the VT assay (Fig. 3a–f). There was an acceptable correlation between the ATPase EC_{50} and VT IC_{50} data (Table 1; Fig. 2) for these six compounds (less than threefold difference). Again, ranking of potencies for ATPase activators was similar in ATPase activation and VT inhibition (itraconazole > ritonavir > verapamil ∼ ketoconazole ∼ quinidine > erythromycin). All compounds which did not activate the ATPase activity but inhibited the verapamil-stimulated ATPase activity also inhibited NMQ transport using the VT assay (Fig. 3g–j). For these ATPase nonactivators (LY335979, PSC833, GF120918, cyclosporin A) a reasonable correlation was seen between ATPase IC_{50} and VT IC_{50} data (Table 1).

Table 1 Interaction of substrates and inhibitors with ABCB1 in ATPase and VT inhibition assays

Compound	ATPase EC_{50} ± SD		ATPase IC_{50} ± SD		VT IC_{50} ± SD	
ABCB1 substrates						
Digoxin	37.5	19.1	212	23.6	284	72.3
Fexofenadine	32.0	17.5	1182	633	235	111
Indinavir	0.913	0.160	16.7	15.1	2.55	0.459
Vincristine	2.67	0.990	56.5	36.6	3.43	2.51
Colchicine	321	80.0	na	na	755	215
Topotecan	na	na	na	na	na	na
Paclitaxel	0.199	0.129	3.42	0.754	1.52	0.0933
ABCB1 inhibitors						
Ritonavir	0.234	0.169	0.287	0.128	0.286	0.0357
Erythromycin	15.8	11.7	498	54.7	38.9	6.17
Quinidine	2.28	1.62	159	24.0	5.41	2.23
Verapamil	2.11	1.49	331	38.5	2.67	0.610
Ketoconazole	3.14	0.735	27.1	6.72	3.86	2.64
Itraconazole	0.0292	0.00571	0.0541	0.0224	0.0485	0.00636
Cyclosporin A	na	na	0.378	0.169	0.115	0.000919
PSC833	na	na	0.0615	0.0346	0.0319	0.0121
GF120918	na	na	0.0560	0.0443	0.153	0.0634
Lilly335979	na	na	0.0122	0.00204	0.0159	0.00255

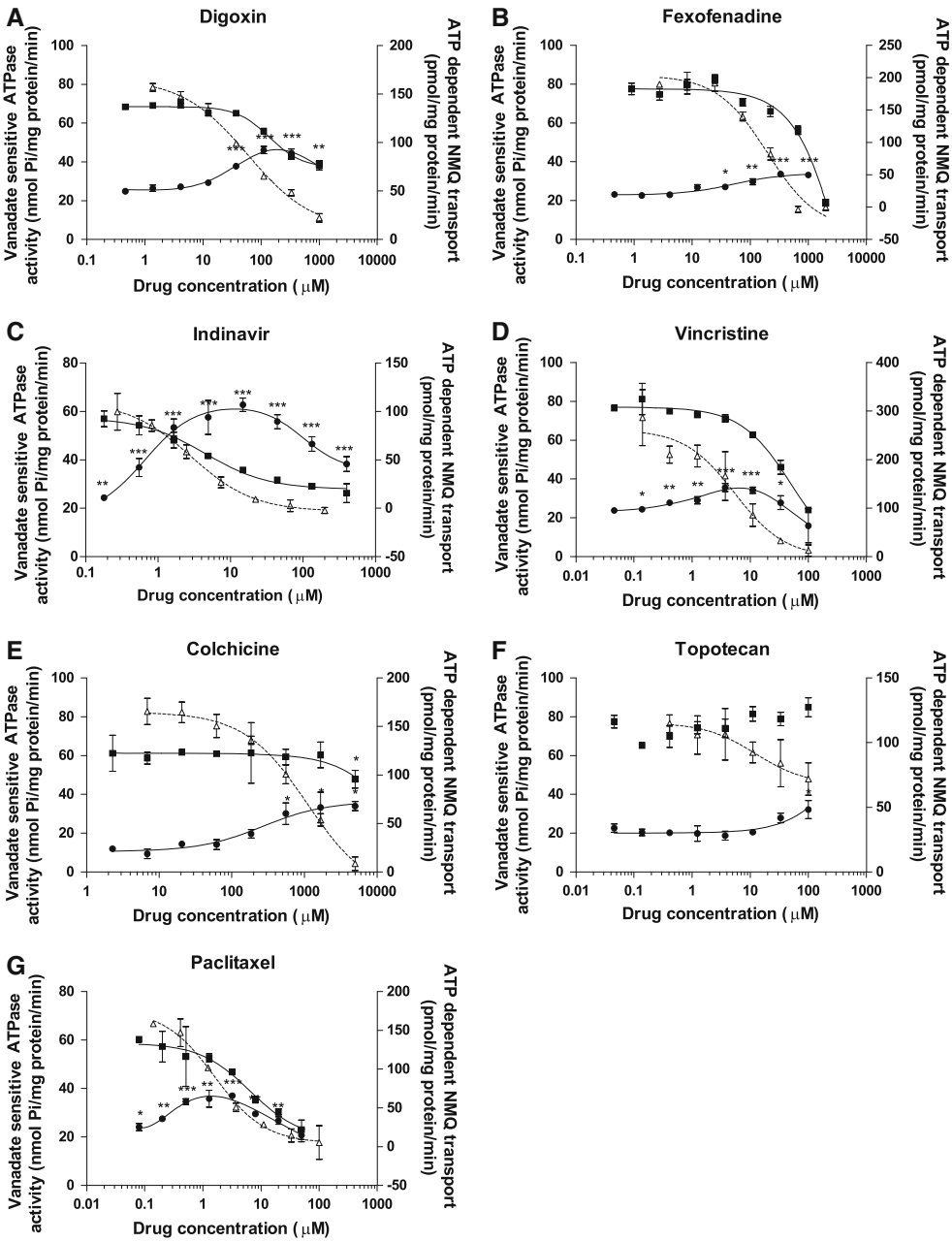

Fig. 1 Dose response of compounds listed as ABCB1 substrates in the ATPase and vesicular transport assay (a–g). Increasing concentrations of compounds listed as ABCB1 substrates were incubated with ABCB1 overexpressing membranes. Vanadate-sensitive ATPase activity in the activation mode (*closed circles*) and in the inhibition mode using verapamil (40 μM) as an activator (*closed squares*) of the ATPase assay as well as ATP-dependent transport of NMQ into inside-out membrane vesicles in the vesicular transport assay (*open triangles*) was measured and plotted as vanadate-sensitive ATPase activity (nmol Pi/mg protein/min) or ATP-dependent transport activity (pmol/mg protein/min)

Discussion

Several studies have shown that ABC transporter ATPase activities are intrinsically linked with substrate transport (Doige et al. 1992). Mutations in the ATPase domain result in loss of transport function (Currier et al. 1989) and the activity of the multidrug transporter in drug-resistant cells is associated with rapid cellular ATP depletion when ATP synthesis is inhibited (Broxterman et al. 1988). Purification and reconstitution of ABCB1 into proteoliposomes also confirmed that ATPase activity and transport (Ambudkar et al. 1992) are linked.

Seven compounds classified as ABCB1 substrates, and ten compounds classified as ABCB1 inhibitors, were tested for both activation and inhibition of ABCB1 ATPase activity (FDA-Guidance 2006). Also, the ability of these compounds to inhibit NMQ transport into ABCB1 expressing vesicles was evaluated. NMQ is one of the few low passive permeability substrates of ABCB1 (Hooiveld et al. 2002). Several established ABCB1 substrates such as digoxin, vinblastine, and paclitaxel non-competitively inhibit NMQ transport by ABCB1 (Heredi-Szabo et al. 2013). Nevertheless, the NMQ assay is a well established assay and has enabled the determination of ABCB1-based

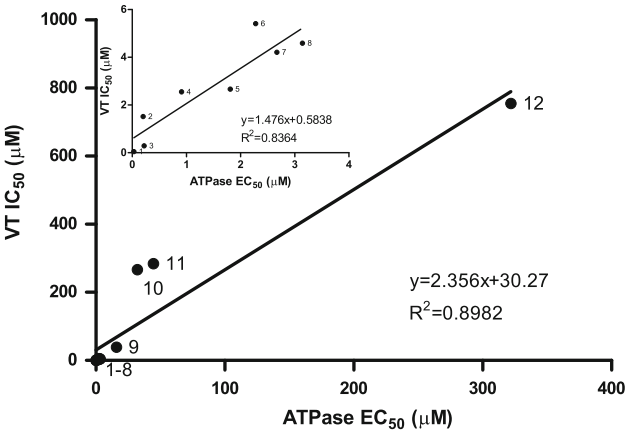

Fig. 2 Correlation between ABCB1 ATPase EC_{50} and ABCB1 VT IC_{50} values For ABCB1 ATPase activators ATPase EC_{50} values and VT IC_{50} values from Table 1 were plotted. The *inset* shows data for the most potent group of compounds. *1* Itraconazole, *2* Paclitaxel, *3* Ritonavir, *4* Indinavir, *5* Verapamil, *6* Quinidine, *7* Vincristine, *8* Ketoconazole, *9* Erythromycin, *10* Fexofenadine, *11* Digoxin, *12* Colchicine

drug interactions for a variety of compounds (Bentz et al. 2013; Heredi-Szabo et al. 2013).

As expected, all compounds previously classified as substrates activated ABCB1 ATPase (Fig. 1a–g; Table 1 and 2). Interestingly, however, six of the compounds (ritonavir, erythromycin, quinidine, verapamil, ketoconazole, itraconazole) classified as inhibitors also activated the ATPase activity (Fig. 3a–f). In previous studies, the efflux ratio of some of the highly permeable compounds (verapamil, ketoconazole, itraconazole) was not modulated by ABCB1 in a bidirectional MDCKII-MDR1 monolayer efflux assay (Polli et al. 2001). However, in another study, verapamil was classified as a transported substrate when the apparent permeability was measured in Caco-2 cells in the A-B direction in the presence and absence of GF120918, a well-known ABCB1 inhibitor (von Richter et al. 2009). In addition, all three compounds displayed ABCB1/Abcb1a dependent brain exposure in vivo (Miyama et al. 1998; Takano et al. 2006;

Table 2 Correlation of ABCB1 ATPase activation data with in vitro and in vivo transport data

Compound	ATPase activation	Transport	
		In vitro	In vivo *S*/*I*
Digoxin	Y	Y (Polli et al. 2001; von Richter et al. 2009)	Y (Ding et al. 2004; Jalava et al. 1997)
Fexofenadine	Y	Y (Petri et al. 2004)	Y (Gupta et al. 2001; Wang et al. 2002)
Indinavir	Y	Y (Hochman et al. 2000)	Y (Kim et al. 1998; van Praag et al. 2000)
Vincristine	Y	Y (Polli et al. 2001)	Y (Mistry et al. 2001)
Colchicine	Y	Y (Polli et al. 2001)	Y (Rollot et al. 2004; Speeg et al. 1992)
Topotecan	Y	Y (Li et al. 2008)	Y (Leggas et al. 2006)
Paclitaxel	Y	Y (Collett et al. 2004)	Y (Kang et al. 2001; Malingre et al. 2001)
Ritonavir	Y	Y (Polli et al. 2001; von Richter et al. 2009)	Y (Shi et al. 2013)/Y (Buss et al. 2001; Ding et al. 2004; Kharasch et al. 2008)
Erythromycin	Y	Y (Polli et al. 2001; von Richter et al. 2009)	Y (Schuetz et al. 1998)/Y (Schwarz et al. 2000)
Quinidine	Y	Y (Polli et al. 2001; von Richter et al. 2009)	Y (Sziraki et al. 2011)/Y (Kharasch et al. 2003; Sadeque et al. 2000)
Verapamil	Y	Y (von Richter et al. 2009)	Y (Bauer et al. 2012)/Y (Sakugawa et al. 2009)
Ketoconazole	Y	N (Polli et al. 2001)	Y (von Moltke et al. 2004)/Y (Simpson and Jarvis 2000)
Itraconazole	Y	N (Polli et al. 2001)	Y (Miyama et al. 1998)/Y (Jalava et al. 1997)
Cyclosporine A	N/Y (Adachi et al. 2001)	Y (Saeki et al. 1993)	Y (Adachi et al. 2001)/Y (Eleftheriou et al. 2008; Meerum Terwogt et al. 1999)
PSC833	N	Y (Smith et al. 1998)	Y (Smith et al. 1998)/Y (Kovarik et al. 1999; Tidefelt et al. 2000)
GF120918	N	N (Polli et al. 2001)	Y (Bauer et al. 2013; Kawamura et al. 2011)/Y (Choo et al. 2006; Huisman et al. 2003)
LY335979	N	N (Dantzig et al. 1996)	Y (Bihorel et al. 2007; Callies et al. 2003)

Data with no literature reference are data from this study

Y yes, *N* no, *S* substrate, *I* inhibitor

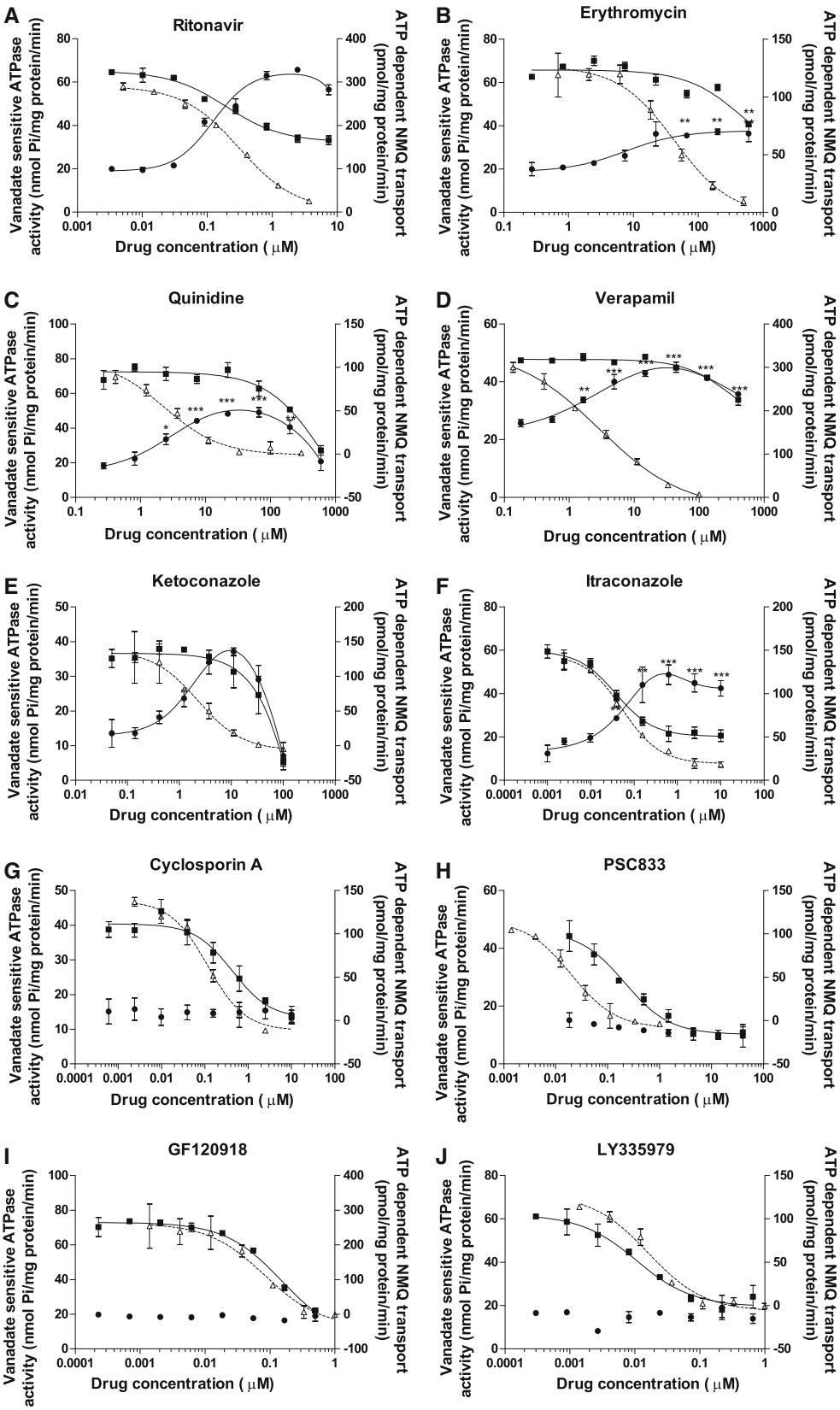

Fig. 3 Dose response of compounds listed as ABCB1 inhibitors in the ATPase and vesicular transport assay (**a**–**j**). Increasing concentrations of compounds listed as ABCB1 inhibitors were incubated with ABCB1 overexpressing membranes. Vanadate-sensitive ATPase activity in the activation mode (*closed circles*) and in the inhibition mode using verapamil (40 μM) as an activator (*closed squares*) of the ATPase assay as well as ATP-dependent transport of NMQ into inside-out membrane vesicles in the vesicular transport assay (*open triangles*) were measured and plotted as vanadate-sensitive ATPase activity (nmol Pi/mg protein/min) or ATP-dependent transport activity (pmol/mg protein/min)

von Moltke et al. 2004). Three of the low-to-intermediate passive permeability compounds classified as inhibitors (ritonavir, erythromycin, and quinidine) also activated the ABCB1 ATPase. The transport of these compounds has been shown previously in monolayer efflux studies (Polli et al. 2001; von Richter et al. 2009). The ABCB1 reversing agents cyclosporin A, PSC833, GF120918, and LY335979 did not activate the ABCB1 ATPase and efficiently inhibited the ATPase activation by verapamil (Fig. 3g–j). As cyclosporin A (Adachi et al. 2001; Polli et al. 2001), PSC833 (Smith et al. 1998), and GF120918 (Bauer et al. 2013; Kawamura et al. 2011) transport by ABCB1 has been demonstrated, this is clearly a lack of correlation between ATPase activation and transport. However, cyclosporin A was reported to be a weak activator of ABCB1 ATPase in another study (Adachi et al. 2001). This latter study used High-Five insect cell membranes overexpressing ABCB1, and it is possible that the difference in expression system may explain the observed activation of ABCB1 ATPase by cyclosporin A. GF120918 displayed an ABCB1-dependent brain permeability in a PET study in mice (Kawamura et al. 2011) as well as in humans (Bauer et al. 2013), and has also appeared to show human ABCB1-dependent permeability in MDCKII-MDR1 cells (Polli et al. 2001), although due to low mass balance in that system it was not classified as a substrate. All the compounds tested inhibited NMQ transport in the VT assay.

The overall correlation between the ATPase activation and transport is around 80 % (Table 2). Two groups of compounds appear to lack correlation across all assays. The high passive permeability inhibitors (ketoconazole, itraconazole) are negative in in vitro transport assays, as the contribution of the transporter to the overall permeability is limited by the high passive permeability of the compound. Some of the inhibitors (cyclosporin A, PSC8333, and GF120918) are negative in ATPase activation assays, but have been shown to be transported substrates in some in vitro and/or in vivo assays. These compounds are considered reversal agents and as such have low off rates, which may limit the signal in the ATPase activation assay.

The ranking of ATPase activation potencies (itraconazole > ritonavir ~ paclitaxel ~ indinavir > vincristine ~ verapamil ~ quinidine > erythromycine ~ fexofenadine ~ digoxin > colchicine ~ topotecan) is similar to ranking of potencies observed in VT inhibition (itraconazole > ritonavir > paclitaxel ~ indinavir > vincristine ~ verapamil ~ quinidine > erythromycin > fexofenadine ~ digoxin > colchicine > topotecan). ABCB1 ATPase EC_{50} values were plotted against ABCB1 VT IC_{50} values for compounds where both values could be calculated, and a reasonable correlation was found, as shown in Fig. 2. It is noteworthy that the VT IC_{50} values are on an average approximately 2.5-fold higher than the EC_{50} values for the whole dataset (Fig. 2) and are approximately 1.5-fold higher than the EC_{50} values for the most potent compounds (Fig. 2 inset). Since VT IC_{50} values depend on the concentration of the probe, Ki values would be more relevant to compare to EC_{50} data. Derivation of K_i values from IC_{50} values is also dependent on the mechanism but for most types of inhibitions the IC_{50}/K_i ratio is greater than 1 (Cer et al. 2009). As the mechanism of NMQ transport inhibition was not determined the Ki values were not computed. In addition, the ATPase data were generated using Sf9 membranes and the VT data were generated in mammalian membranes potentially confounding correlations as membrane lipid composition has a profound effect on ABCB1 activity (Clay and Sharom 2013; Heredi-Szabo et al. 2013), although the effect of membrane lipid composition on IC_{50} values of inhibitors has not been investigated.

For compounds that were ATPase non-activators such as LY335979, PSC833, GF120918, cyclosporin A a reasonable correlation was seen between ATPase IC_{50} and VT IC_{50} data (Table 1). For the ATPase activators, VT IC_{50} values showed no correlation with ATPase IC_{50} values for most compounds (Table 1). Hence, significance of ATPase inhibition data is low.

Application of ATPase activation data may be limited to qualitative (yes/no) answers, as the single published study showed little correlation between human ABCB1 ATPase activation and murine Abcb1a function at the blood–brain barrier (Adachi et al. 2001). Nevertheless, it is clear that ATPase data for high passive permeability compounds in particular, aids correct classification (substrate vs inhibitor) of transporter interactors. The difference between the ATPase-based classification presented here and the classification reported (FDA-Guidance 2006) is most likely due to the fact that pharmaceutical classification is mostly based on clinical data. When in vitro data are considered the cut-off value for a pharmaceutically significant drug–transporter interaction is transfectant/control ≥ 2 albeit a statistically significant increase as cut-off has also been suggested for some transport assays (Giacomini et al. 2010). Based on the transfectant/control ≥ 2 criterium verapamil has been classified as a non-transported substrate (Polli et al. 2001). However, more recently it has been shown that verapamil displays an ABCB1-dependent brain exposure in humans (Bauer et al. 2014; Muzi et al. 2009; Romermann et al. 2013), as suggested by the ATPase activation data (Fig. 3d).

Most importantly, all compounds tested have shown interaction with ABCB1 ATPase activation and/or inhibition assays. All interactions were detected in the VT inhibition assay. In addition, all compounds that activated the ATPase are known substrates of the transporter.

Six of ten compounds classified a priori as inhibitors activated the ATPase of ABCB1. With the exception of cyclosporin A, the only compounds classified as inhibitors that did not activate the respective ATPase were reversal agents, developed to inhibit ABCB1 (Lehnert et al. 1993). Therefore, it is highly recommended to use reversal agents as reference inhibitors. For ABCB1, specific inhibitors exist and PSC833 is commercially available (Sziraki et al. 2011).

In sum, membrane assays are good high throughput tools to measure drug–transporter interactions and provide a ranking of the potencies of these interactions.

Acknowledgments The help of Timea Rosta, MSc in preparation of the manuscript is acknowledged. The work was supported by the following Hungarian Grants: GOP-1.1.1-11-2011-0064, GOP-1.1.1-11-2011-0017 (PRODRUG), FP7 EUSTROKE, HEALTH-F2-2008-202213, XTTPSRT1, OM-00230/2005, TUDAS-1-2006-0029, OMFB-00505/2007, GOP-1.1.1-09/1-2009-0054.

Conflict of interest SOLVO Biotechnology and Xenotech LLC specialize in development and commercialization of transporter technology applications.

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