Comparison of in Vitro P-Glycoprotein Screening Assays: Recommendations for Their Use in Drug Discovery

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The ATP-dependent drug efflux pump P-glycoprotein (P-gp) affects the absorption and disposition of many compounds. P-gp may also play role in clinically significant drug−drug interactions. Therefore, it is important to find potential substrates or inhibitors of P-gp early in the drug discovery process. To identify compounds that interact with this transporter, several P-gp assays were validated and compared by testing a set of 28 reference compounds, including inhibitors of cytochrome P450 3A4 (CYP3A4). The assays included in silico predictions, inhibition assays (based on cellular uptake of rhodamine-123 or calcein AM), and functional assays (ATPase activity assay and transcellular transport assay, the latter for a subset of compounds). In addition, species differences were studied in an indirect fluorescence indicator screening assay and test systems expressing porcine, mouse, or human P-gp. Our results suggest that several P-gp assays should be used in combination to classify compounds as substrates or inhibitors of P-gp. Recommendations are given on screening strategies which can be applied to different phases of the drug discovery and development process.

Introduction

P-glycoprotein (P-gp) is a 170-kDa membrane protein that functions as an energy-dependent efflux pump. P-gp is a member of the ATP binding cassette (ABC) superfamily of secretory transport proteins.[1] P-gp is encoded by the MDR1 gene in humans.[2] Mice and other analyzed rodents have two drug-transporting P-glycoprotein genes, mdr1a (also called mdr3) and mdr1b (also called mdr1).[3] A large number of structurally diverse and unrelated agents are substrates for P-gp.[4] In laboratory models, overexpression of P-gp can confer resistance to structurally dissimilar cytotoxic drugs such as vinca alcaloids, anthracyclines, taxoids, and actinomycin D by decreasing intracellular drug concentration. The overexpression of P-gp results in a well-characterized mechanism of chemoresistance known as multidrug resistance.[5]

P-gp has been localized in several human tissues including the liver, kidney, gastrointestinal tract, and blood−brain barrier.[6,7] P-gp affects the pharmacokinetics of many drugs, and its effects have been demonstrated in vivo by means of mdr1a and mdr1b knockout mice,[8] and inhibitors of P-gp such as PSC 833, a nonimmunosuppressive cyclosporin.[9] In particular, its impact on intestinal absorption[10] and blood-brain barrier permeability[11] have been discussed.

The clinical relevance of the effects of P-gp on drug absorption has been demonstrated by recent clinical studies that suggest that there are functional polymorphisms of human MDR1 in intestinal absorption,[12] although the issue is controversial.[13,14] There are many instances of clinical drug−drug interactions, which result from inhibition or induction of P-gp.[15] Examples of such clinical drug−drug interactions, which are likely to be caused by P-gp, include decreased digoxin levels in patients treated with rifampin leading to reduced oral bioavailability of digoxin due to induction of intestinal P-gp,[16] likely interactions between cyclosporine and the calcium antagonist felodipine,[17,18] clinically significant pharmacokinetic interactions between digoxin and vincristine, erythromycin, quinidine, verapamil, and nifedipine[19] and reversal of clinical multidrug resistance by verapamil, cyclosporin,[20] or the P-gp inhibitor PSC 833.[21] Furthermore, the marked increase in the plasma concentrations of protease inhibitors when coadministered with ritonavir[22] might be, at least in part, caused by P-gp drug interactions during oral absorption. Such antiretroviral combination treatment regimens improve neurocognitive symptoms in HIV infection and substantially lower CSF viral load[23] and thus suggest clinical significance of P-gp mediated drug−drug interactions at the level of the blood−brain barrier.

The clinical relevance of the effects of P-gp on brain penetration has been demonstrated by preclinical studies. Differences in drug concentrations in brain relative to plasma observed in P-gp KO (mdr1a or mdr1a/1b knock-out mice) and P-gp competent mice correlate with transport data from mdr1a-expressing cell lines.[24,25] Species differences in P-gp transport and thus brain penetration were suggested by the same authors by using P-gp transport data from mdr1a and MDR1 expressing cells. The relevance of P-gp activity in limiting accessibility of P-gp substrates to the brain is further supported by pharmacological animal studies.[26−29] These studies demonstrate that reduction of P-gp efflux at the blood−brain barrier increases the pharmacological activity of P-gp substrates.

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Several in vitro screening assays have been used to identify and classify compounds as P-gp substrates or inhibitors. For example, drug-stimulated ATPase activity can be determined by monitoring the release of inorganic phosphate by a colorimetric reaction.[30,31] Another approach is the use of fluorescent indicators[32,33] where inhibition of P-gp leads to cellular accumulation of a fluorescent dye, such as rhodamine-123[34] or calcein AM.[35] Such fluorometric assays may be automated because they produce a readout that is suitable for high throughput screening. However, unambiguous identification of inhibitors or substrates of P-gp may be difficult due to the presence of multiple binding sites. Kinetic data indicating noncompetitive interactions between substrates and inhibitors of P-gp,[36,37] as well as photoaffinity labeling studies,[38] suggest the presence of at least two distinct substrate binding sites. The two sites have distinct but overlapping substrate specificities and exhibit positive cooperativity. A third drug-binding (nontransporting) site with positive allosteric properties for drug transport has also been reported.[39] Thus, inhibition data obtained from P-gp interaction studies may depend on the substrate used, as suggested for drug interaction at cytochrome P450 3A4 (CYP3A4).[40]

Species differences with respect to substrate and inhibitor specificity among P-glycoproteins encoded by human MDR1 or mouse mdr1a and mdr1b genes were reported recently.[41,42] Therefore test systems that are applied to P-glycoproteins derived from different species are likely to reflect these species differences.

Little is known about possible polymorphisms in the P-gp protein expressed in test systems. P-gp activity seems to be particularly sensitive to the nature and physical state of the surrounding lipids.[31] Thus, the particular in vitro model used may influence the kinetic properties of P-gp.

Compounds can be classified as inhibitors and/or substrates of P-glycoprotein.[43] It is important to note that some P-gp substrates, which can be identified in ATPase assays, do not undergo significant membrane transport. This apparent lack of P-gp transport is observed for compounds with a high passive permeability such as midazolam, nifedipine, and verapamil,[44] where a fast transmembrane flux rate may overcome P-gp-mediated efflux.[34,45]

The aim of our study was to compare and validate several types of P-gp assays. We used a human P-gp ATPase assay to evaluate P-gp activity.[30] For selected compounds, P-gp activity was measured by a direct transport assay, using polarized LLC−PK1 epithelial cells transfected with mdr1a or MDR1.[46] Inhibition of P-gp was studied in two fluorescence indicator assays: One assay used the fluorescent dye calcein AM in polarized LLC−PK1 epithelial cells transfected with mdr1a or mdr1b or MDR1.[46] Calcein AM was also used in primary cultures of porcine brain capillary endothelial cells.[47] The other assay used the fluorescent dye rhodamine-123 in a vinblastine-resistant Caco-2 cell line.[48] Twenty-eight reference compounds were selected. All compounds were either known from the literature to interact with P-gp, predicted by in silico modeling[4] to be substrates or inhibitors of P-gp, or metabolized by CYP3A4, an enzyme that contributes to phase I metabolism in humans and has been shown to have overlapping substrate specificities with P-gp.[49,50]

Materials and Methods

Materials. Human P-gp membranes (MDR1) were purchased from Gentest Corporation (Woburm, MA).

Cell Cultures. Low passage (27) Caco-2 cells (HTB-37) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle Media (DMEM) with high glucose and L-glutamine supplemented with 10% fetal bovine serum, penicillin−streptomycin (100 IU/100 μg per mL), and 0.1 mM nonessential amino acids (all Gibco/Life Technologies, Carlsbad, CA). P-gp induction was performed by subculturing in the above media supplemented with 20 nM vinblastine (Sigma, St. Louis, MO). Cells were harvested by trypsinisation 96 h prior to the experiment, washed, and resuspended in the above media without the vinblastine at a density of 1.5×10^5 cells/mL. BIOCOAT poly-D-lysine-coated 96-well black/clear plates (Becton Dickinson, Franklin Lakes, NJ) were seeded with 200 μL of the cell suspension (30000 cells/well) and maintained at 37 °C and 5% CO_2 for 96 h to allow complete washout of vinblastine and reconditioning of the cells. Cells from passage 34−45 were used for the present study.

Primary cultures of porcine brain capillary endothelial cells were prepared and cultured as described previously.[51] Cells were seeded at 120 000 cells/well in 24-well tissue culture plates coated with collagen. Confluent cell monolayers were used at day 4 in culture for the fluorescence indicator assay.

The pig kidney epithelial cell lines L-mdr1a and L-mdr1b (mouse transfectants) and L-MDR1 (human transfectant) derived from LLC−PK1 cells were obtained from Dr. A. Schinkel, The Netherlands Cancer Institute (Amsterdam, The Netherlands) and used under license agreement. Cells were seeded at 10^6 cells/flask in 50 cm^2 cell culture flasks and were cultured as described.[52] Cell culture medium consisted of Medium 199 with Glutamax (Gibco/Life Technologies) supplemented with 10% fetal bovine serum and 5 mL penicillin/streptomycin (10 000 IU penicillin and 10 mg streptomycin per mL) per 500 mL medium. Subcultures were obtained once a week from confluent cell monolayers using a split ratio of 1:10−1:20. Cells were subcultured in the presence of 640 nM vincristine (transport experiments) or 150 ng/mL colchicine (fluorescence indicator assays) to maintain P-gp expression. Twenty-four hours prior to transport experiments, cells were transferred to a medium without vincristine. Prior to the experiment, L-mdr1a or L-mdr1b or L-MDR1 cells were detached by trypsinisation (10 min. using EDTA/trypsin, Gibco/Life Technologies), washed with assay buffer (D-MEM/F12, Gibco/Life Technologies) and transferred to 24-well cell culture plates at a cell density of 100 000 cells/well. Cell suspensions were used within 3 h for the fluorescence indicator assays.

LLC−PK1 derived cell lines were passaged at least three times before using them for experiments. Once in culture and induced, cells have been shown to express constant levels of P-gp for up to 15 passages.[44] Cells from passage 15−30 were used for the present study.

Assays. Pharmacophore models for in silico predictions of P-gp substrates as well as P-gp modulators were generated using CATALYST (Accelerys, San Diego, CA). A set of 30 reference P-gp substrates and 30 P-gp modulators independent from the data set reported in this study were used in the identification of common functional 3D features of each group. The hypothesis found for P-gp substrates consists of a 3D arrangement of two hydrogen-bond acceptors with a spatial separation of 6.62−8.62 Å and a hydrophobic group. The hypothesis found for P-gp modulators consists of a fixed spatial arrangement of three hydrophobic moieties and a hydrogen-bond acceptor group. A similar P-gp pharmacophore model has been described recently by Ekins et al. using the same approach.[53]

Cytochrome P450 3A4 (CYP3A4) inhibition was determined by a fluorescent indicator assay according to a published procedure[54] using the CYP3A4 substrate 7-benzyloxy-4-(tri-

fluoromethyl)coumarin (BFC). The dose−response relationship of inhibition was determined using a range of concentrations of test compound (0.01 to 50 μM).

1. Rhodamine Assay. Caco-2 cells were washed and media was replaced by DMEM containing 5% FBS. Test compounds were dissolved in DMSO at a concentration of 10 mM. A working solution of 100 μM test compound was prepared in media. Caco-2 cells were incubated with test compounds at a final concentration of 50 μM (final DMSO concentration of 0.5% for all incubations). The concentration of test compound may be lowered according to expected systemic exposure in vivo. Cells were preincubated for 15 min at 37 °C and 5% CO_2, before rhodamine was added at a final concentration of 1 μg/mL (2.6 μM). Cells were then incubated for 4 h at 37 °C, washed twice with ice-cold PBS, and scanned using a Cyto-Flour fluorescence multiwell plate reader (PerSeptive Biosystems by Applied Biosystems, Foster City, CA) at an excitation wavelength of 450 nm and an emission wavelength of 530 nm. A possible interference due to the auto fluorescence of all test compounds was evaluated. Results were expressed as % inhibition of P-gp relative to the positive control nicardipine (100% inhibition).

2. Calcein AM Assay.[35] Cells of different origin in 24-well plates (i.e., cell suspensions of L-mdr1a, L-mdr1b, or L-MDR1 cells or confluent monolayers of porcine brain capillary endothelial cells in assay buffer (D-MEM/F12, Gibco/Life Technologies)) were incubated with a range of concentrations (0.05 to 50 μM) of test compound at 37 °C for 10 min. Stock solutions (10 mM) of compound were prepared in DMSO. This concentration was reduced to 2 mM for compounds showing low solubility in DMSO such as itraconazole, midazolam, and terfenadine. However, final concentration of the solvent DMSO in the assay was kept constant at 1%. Calcein AM (Molecular Probes, Eugene, OR) was added to give a final concentration of 0.25 μM. Plates were immediately placed in a TECAN fluorescence plate reader (Spectrafluor plus, TECAN, Männedorf, Switzerland), and calcein fluorescence was continuously monitored at 37 °C for 45 min at 458 nm excitation and 520 nm emission wavelength. One well on each plate contained the internal standard (positive control) verapamil (50 μM). From the time-dependent increase of cellular fluorescence the initial rate of fluorescence generation (IRF) was determined. Quantification of P-gp inhibition was done using the following equation: % inhibition = (IRF(test compound)-IRF(background))/(IRF(verapamil) − IRF(background)) × 100 where IRF(verapamil) was the IRF in the presence of 50 μM verapamil and IRF(background) was the IRF in the absence of test compound. A compound was determined to inhibit P-gp when an inhibition of ≥50% was reached. In that case, the extrapolated concentration at which inhibition was 50% was reported and defined as IC_{50} value. The assay was partially automatized on a TECAN pipetting robot (Genesis Workstation 200, TECAN) to increase the assay throughput.

3. ATPase Assay.[30] Human P-gp membranes (MDR1) (40 μg in 60 μL incubation volume) were incubated in the presence of 20 μM verapamil (positive control), 0.5% DMSO (negative control), or test compound (20 μM), and 4 mM MgATP in buffer (50 mM Tris-MES, 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol, 5 mM sodium azide) at 37 °C for 20 min. An identical reaction mixture containing 100 μM sodium orthovanadate, a selective inhibitor of the P-gp ATPase, was assayed in parallel. Thus, ATPase activity measured in the presence of orthovanadate represents non-P-gp ATPase activity and can be subtracted from the activity generated without orthovanadate to yield vanadate-sensitive ATPase activity. The reaction was stopped by the addition of SDS (3%). Two additional reaction mixtures (with and without orthovanadate) but without MgATP, were also prepared to represent the initial conditions of the reaction. Liberation of inorganic phosphate was monitored by the addition of 2 volumes of 35 mM ammonium molybdate in 15 mM zinc acetate: 10% ascorbic acid (1:4, v/w) and incubation for 20 min at 37 °C. The inorganic phosphate complex was detected by its absorbance at 800 nm and quantitated by comparing the absorbance to a phosphate standard curve after subtraction of the signal obtained by the negative control (i.e., 0.5% DMSO). P-gp ATPase activity greater than 1.2 nmol/mg/min (Table 4) was significant because it corresponded to the 98% confidence level of mean background activity.

4. Transcellular Transport Assay. LLC−PK1, L-MDR1, and L-mdr1a cells were seeded at a density of 1.4×10^5 cells/well on porous poly(ethylene terephthalate) membrane filters (3 μm pore size, 0.31 cm^2 filter area, Falcon) which were mounted in Falcon 24-well plates. Cells were used for the transcellular transport study 4 days after seeding. The medium of each cell monolayer was replaced with fresh medium without vincristine 1 day before the experiment. Transcellular transport experiments were initiated by adding test compound to the apical (300 μL) or basal (700 μL) side together with either [^{3}H]inulin (0.5 μCi/ml) or [^{14}C]inulin (0.25 μCi/ml) as extracellular marker. The specific activity of radiolabeled test compound was 3 μCi/mL corresponding to compound concentrations of less than 350 nM. Incubations were done in culture medium at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. Samples were taken from the acceptor compartment at 1, 2, 3, and 6 h and analyzed by liquid scintillation counting. Transport experiments were performed in the presence of 10% fetal calf serum (FCS) which increases solubility of poorly soluble drugs and may decrease unspecific binding compared to experimental conditions without FCS. It is nevertheless recommended to determine recovery of the compounds tested.

The transport ratios across the monolayers were calculated for each cell line separately as described.[25] Briefly, apparent permeabilities (P_{app}) were calculated according to: $P_{app} = dQ/dt \times 1/A/C_0$ (cm min^{-1}), where dQ/dt is the initial rate of translocation (calculated from a linear regression), A is the surface area of the filter membrane, and C_0 is the initial concentration of the labeled drug. Transcellular transport ratios were expressed as apparent permeabilities obtained in the basolateral-to-apical direction divided by those obtained in the apical-to-basolateral direction. Corrected transcellular transport ratios were calculated as the transport ratio obtained in either MDR1 or mdr1a cells, divided by the transport ratio obtained in parental LLC−PK1 cells. Radiolabeled inulin was used as a paracellular flux marker. Experiments where >0.7%/h inulin was transported were rejected.

Results and Discussion

Many aspects of P-glycoprotein-mediated substrate recognition and transport are poorly understood, and the underlying mechanisms seem to be complex.[43,55] As a consequence, the predictive value as well as the limitations of in vitro screening assays used for the identification and classification of substrates or inhibitors of P-gp have to be assessed carefully. It was the aim of the present project to validate several P-gp screening assays with a set of 28 reference compounds (Table 1). The test compounds were structurally diverse, covered a wide range of physicochemical properties (molecular weight and measured or calculated octanol to water partition coefficients $clogP_{o/w}$), and had been described in the literature to be either substrates and/or inhibitors of P-gp or cytochrome P450 3A4 (CYP3A4).

In Silico Predictions and CYP3A4. All compounds except morphine showed a potential to interact with P-gp in an in silico pharmacophore model. CYP3A4 interactions were considered because several authors[10,49,50] have proposed overlapping substrate specificities or a combined role of CYP3A4 and P-gp. Our results indicate some overlaps, but also demonstrate distinct substrate specificities for the two enzyme systems. For example, digoxin is metabolically stable,[56] but has been shown in vivo to be a substrate of P-gp.[57]

Table 1. Validation Set, in Silico Prediction of P-gp Interactions, Physicochemical Properties, and CYP3A4 Inhibition[a]

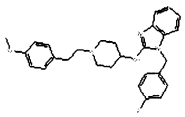
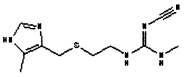
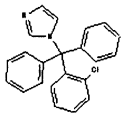
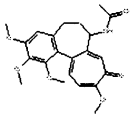
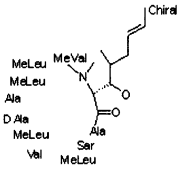
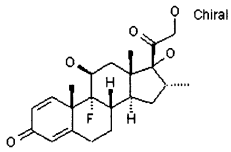
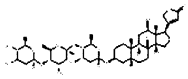
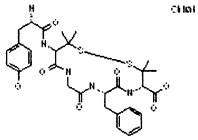
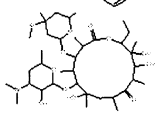
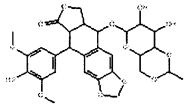
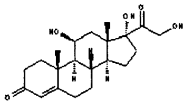
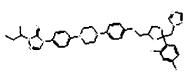
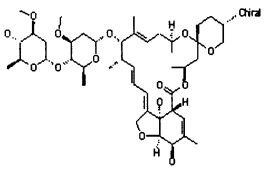
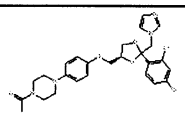
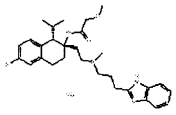
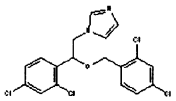
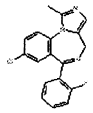
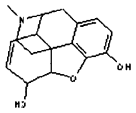
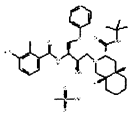
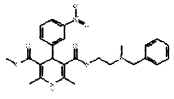
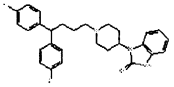
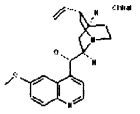
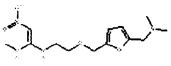
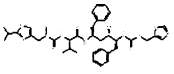
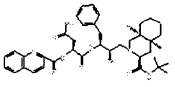
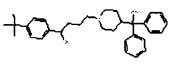
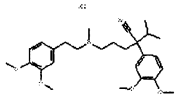
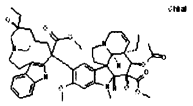
Compound	Chemical Structure	in silico substrate	in silico modulator	MW	logP o/w	IC_{50} (CYP3A4) [μM]	In vivo classification (Reference)
Astemizole		X	X	458	5.7	3.3	
Cimetidine		X	X	252	0.4	15	
Clotrimazole		-	X	344	5.3 [c])	0.02	
Colchicine		X	X	399	1.3	>50	substrate (65)
Cyclosporin A		X	X	1188	2.9	6.5	substrate (52)
Dexamethasone		X	X	392	1.7	> 50 [a]	weak substrate (52)
Digoxin		X	X	780	1.3	>50	substrate (52, 57)
Enkephalin (DPDPE)		X	X	646	1.0 [c])	> 50	
Erythromycin		X	X	399	2.5	33	inhibitor (67)
Etoposide		X	X	588	0.6	>50	
Hydrocortisone		X	X	362	1.6	>50	
Itraconazole		X	X	705	6.5 [c])	0.70	
Ivermectin		X	X	875	4.0 [c])	>50	substrate (66)

Table 1 (Continued)

Compound	Chemical Structure	In silico substrate	In silico modulator	MW	$logP_{o:w}$	IC_{50} (CYP3A4) [μM]	In vivo classification (Reference)
Ketoconazole		X	X	531	3.7	0.05	
Mibefradil		X	X	495	6.2 [a]	0.13	
Miconazole		X	X	416	5.8 [a]	0.18	
Midazolam		-	X	325	3.3	8.1	
Morphin		-	-	285	0.8	>50	weak substrate (52)
Nelfinavir		X	X	567	5.8 [a]	0.55	substrate (64)
Nicardipine		X	X	479	5.5 [a]	0.42	
Pimozide		-	X	461	6.3	9.7	
Quinidine		X	X	324	2.8 [a]	> 50	inhibitor (15)
Ranitidine		X	X	314	0.6 [a]	>50	
Ritonavir		X	X	720	4.9 [a]	0.03	
Saquinavir		X	X	670	4.7 [a]	1.5	substrate (64)
Terfenadine		X	X	471	5.7	0.32	
Verapamil		X	X	454	3.8	4.7	inhibitor (63)
Vinblastine		X	X	811	5.2 [a]	26.0	substrate (42)

[a] Calculated with ClogP v4.71 (Biobyte Inc.).

Table 2. Comparison of in Vitro P-gp Screening Assays

compound	ATPase-assay MDR1 membranes (human), nmol/mg/min	rhodamine-assay CaCo-2 (human) % inhibition	calcein-AM assay PBCEC (porcine) IC_{50} (μM)	calcein-AM assay L-mdr1a (mouse) IC_{50} (μM)	calcein-AM assay L-mdr1b (mouse) IC_{50} (μM)	calcein-AM assay L-MDR1 (human) IC_{50} (μM)	indirect assay classification[a]
astemizole	18	39	0.3	1.3	1.7	1.3	+++
cimetidine	2.8	4	>50	>50	>50	>50	+−−
clotrimazole	7.5	29	1.3	4.8	3.5	6.7	+++
colchicine	0.1	8	>50	>50	>50	>50	−−−
cyclosporin a	9.8	43	0.5	4.8	0.7	0.8	+++
dexamethasone	4.0	0	>50	>50	>50	>50	+−−
digoxin	0.3	1	>50	>50	>50	>50	−−−
enkephalin (dpdpe)	4.0	0	>50	>50	>50	>50	+−−
erythromycin	4.0	1	43	>50	>35	>50	+−−
etoposide	−0.2	1	>50	>50	>50	>50	−−−
hydrocortisone	0.7	0	>50	>50	>50	>50	−−−
itraconazole	18	2	0.03	0.2	0.7	2.1	+−+
ivermectin	−3.4	34	0.2	0.5	0.5	0.1	−++
ketoconazole	27	35	1.0	3.8	6.7	4.8	+++
mibefradil	8.9	60	1.5	7.4	10	1.8	+++
miconazole	1.0	22	2.0	7.8	2.0	3.5	−++
midazolam	6.0	12	>50	>50	33	>50	+−−
morphin	3.7	2	>50	>50	>50	>50	+−−
nelfinavir	6.2	30	0.35	2.3		3.4	+++
nicardipine	18	100	0.95	2.5	8.0	2.3	+++
pimozide	11	37	0.80	4.9	>50	2.9	+++
quinidine	20	36	2.2	13	10	5.6	+++
ranitidine	−2.4	0	>50	>50	>50	>50	−−−
ritonavir	19	116	1.5	50	>50	12	+++
saquinavir	30	43	1.6	>50	>50	12	+++
terfenadine	0.4	6	1.1	23	2.0	1.4	−−+
verapamil	21	28	0.4	10	2.0	6.3	+++
vinblastine	33	75	2.0	>50	8.0	>50	+++

[a] The first symbol of the classification stands for positive (+) or negative (−) testing in the functional ATPase assay, the second symbol stands for + or − testing in the rhodamine assay, the third symbol stands for + or − testing in one of the calcein-AM screening assays.

Quinidine and ivermectin tested negative in the CYP3A4 assay, but were identified to interact with P-gp.

Fluorescent Indicator Assays. Two fluorescence indicator assays in combination with five cell types were used as a primary screen for the identification of substrates and/or inhibitors of P-gp.

A P-gp screening assay, amenable to high throughput, was established based on the observation that accumulation of rhodamine-123 in Caco-2 cells expressing human P-gp can be enhanced in the presence of inhibitors of P-gp.[34] Caco-2 cells were cultured in the presence of 20 nM vinblastine to reduce the variability of P-gp expression and to increase the sensitivity of the assay. Cellular fluorescence was determined after 4 h of incubation in the presence of rhodamine-123 and a single concentration ($\leq$50 μM) of test compound. To obtain a high throughput capacity and facilitate automation this assay was designed in a 96-well plate format. In addition, the endpoint only of rhodamine accumulation in Caco-2 cells was determined. Results were expressed as % inhibition of P-gp relative to the positive control nicardipine (100%). This assay provided an efficient method to rank inhibitors of P-gp (Table 2). Note that rhodamine-123 is a substrate of the renal organic cation carrier,[58] and thus limiting its use to cell lines such as Caco-2, which do not express this carrier.[59]

The other fluorescence indicator assay used calcein AM (Table 2). Calcein AM is a P-gp substrate, and its cellular accumulation is enhanced in the presence of inhibitors of P-gp. Cleavage of the nonfluorescent calcein AM to calcein by intracellular esterases produces a fluorescence signal in the incubation mix. This assay used confluent cell monolayers, such as primary cultures of porcine brain capillary endothelial cells or L-MDR1 cells, grown on a solid support, such as primary cultures of porcine brain capillary endothelial cells or L-MDR1 cells, or cell suspensions such as L-mdr1a or L-mdr1b cells (Table 2). Initial rates of calcein production were determined by continuous monitoring of incubations in the presence of different concentrations of test compound. The IC_{50} value for a given compound was calculated from dose−response curves (Figure 1). While more time-consuming, this procedure allowed us to detect nonlinearity in the rate of fluorescence formation and evaluate the dose−response relationship. A decrease in fluorescence occurred at elevated concentrations of pimozide, cyclosporin A, and ivermectin (Figure 1). An explanation for this phenomenon is the inhibition of esterases. Inhibition of esterases has been observed for high concentrations of indomethacin.[48]

There was a good correlation between the results of the rhodamine assay with vinblastine-induced Caco-2 cells (human origin) and the calcein AM assay with human L-MDR1 cells (Table 2). However, terfenadine and itraconazole were identified as P-gp substrate or inhibitor in the calcein AM assay, but not in the rhodamine assay. This observation might be due to a lack of competitive interaction with the rhodamine 123.[36] It has been suggested that P-gp contains multiple binding sites. For example, colchicine inhibits transport of Hoechst 33342 but stimulates rhodamine-123 transport.[39]

Species Differences. The calcein AM fluorescence indicator assay was used with primary cultures of porcine brain capillary endothelial cells (the main constituents of an in vitro model of the blood−brain barrier[47]) and kidney epithelial LLC−PK1 cells transfected with mouse mdr1a or mdr1b or human MDR1. There was a very good correlation between results obtained using cells expressing porcine or human P-gp (Table 2). Ritonavir, saquinavir, and verapamil tested positive in both systems but had slightly lower IC_{50}

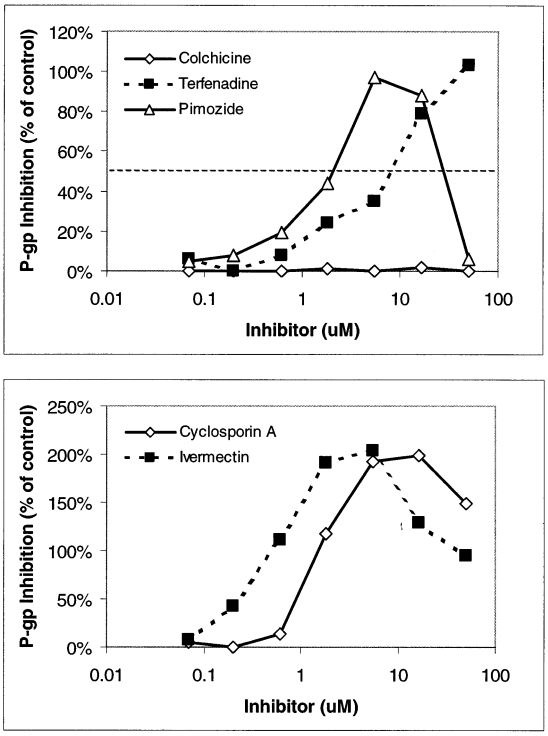


Figure 1. Dose−response of test compounds in the calcein AM assay (L-MDR1): 100% inhibition of P-gp is defined as the signal generated by the internal standard (50 μM of verapamil). The IC_{50} value is defined as the concentration of inhibitor which is necessary to obtain 50% inhibition (dotted horizontal line). Note that some test compounds (lower panel) showed a higher efficacy than verapamil. At high substrate concentrations, a decrease in calcein signal may occur due to precipitation and/or cytotoxicity of test compound. Data of selected single experiments, datapoints are means of two measurements.

values in porcine brain capillary endothelial cells. Vinblastine was identified as an inhibitor of P-gp in porcine cells but not in L-MDR1 cells. This correlation was not observed for mouse P-gp (mdr1a): ritonavir and saquinavir tested negative in L-mdr1a cells but were strong inhibitors of human P-gp or porcine P-gp.[60] Cyclosporin A, mibefradil, miconazole, quinidine, terfenadine, and verapamil were less potent inhibitors for mouse P-gp (mdr1a) as compared to porcine or human P-gp. Such marked differences between MDR1 and mdr1a have also been suggested in recent studies using in vitro transport assays in combination with L-MDR1 and L-mdr1a cells.[42,24] However, it has been unclear if these results might reflect true species differences [24] or if they reflect solely the absence of mdr1b in these test systems since the mouse P-gp isoforms mdr1a and mdr1b have been shown to possess distinct functional characteristics.[41] We therefore decided to include L-mdr1b cells in our study. Our results (Table 2) indicate clearly that mdr1a and mdr1b have distinct and complementary substrate specificities. Cyclosporine A, miconazole, terfenadine, verapamil, and vinblastine show a significantly higher affinity toward mdr1b. Pimozide is an inhibitor of mdr1a but does not interact with mdr1b. The implications of these results are 2-fold: First, L-mdr1a and L-mdr1b screening assays should be done in parallel to identify inhibitors of rodent P-gp. Second, there are species differences between rodent and human P-gp (e.g., ritonavir, saquinavir). However, the differences are smaller than expected based on published studies where only mdr1a was considered. The use of mice deficient for either mdr1a[52] or both mdr1a and mdr1b[8] might provide an opportunity to clarify the roles of mdr1a and mdr1b in vivo.

P-Glycoprotein ATPase Assay. P-gp ATPase activity was measured in the presence of 20 μM of test compound. The 20 μM concentration was chosen because previous studies demonstrated that at that concentration there was ATPase activation for most compounds.[61,44] The interassay variability of the non P-gp ATPase activity was 14.2 ± 0.5 nmol/mg/min (mean ± SEM, n = 31). The final concentration of DMSO in the incubation mix was kept constant at 0.5%. This is important because DMSO stimulates ATPase activity. Incubation with 0.5% and 5% DMSO produced background signals of 8.3 ± 0.1 and 14.0 ± 1.9 nmol/min/mg, respectively. Data represent P-gp ATPase activity after correction of this background signal (0.5% DMSO). P-gp ATPase activities above 1.2 nmol/mg/min (Table 4) were considered to be significant since this corresponds to the 98% confidence level of mean background activity.

Classification of Test Compounds by in Vitro Screening Assays. Based on the in vitro screening assays, a classification for test compounds was established (Table 2) where the first of three symbols stands for positive ("+") or negative ("−") results in the ATPase assay, the second stands for positive or negative results in rhodamine assay, and the third stands for positive or negative results in the calcein AM assay with any of the cell lines. Several P-gp substrates such as vincristine, colchicine, and methotrexate do not stimulate P-gp ATPase activity.[61] This was confirmed for colchicine and was also suggested for digoxin and ivermectin in the present study. Presently, there is no explanation for this phenomenon. This apparent inhibition of P-gp activity may result from a disturbance in the lipid environment by membrane active compounds,[31] limited solubility of test compounds, or direct inhibition of ATPase activity at high concentrations of test compounds. For this reason, compounds with a "− + +" or "− − +" classification, such as ivermectin, miconazole, and terfenadine were retested in the P-gp ATPase assay over a range of substrate concentrations (0.1−20 μM). Data from these studies confirmed the negative result in the initial screen. It is interesting that ivermectin and ranitidine showed negative ATPase results over a wide range of drug concentrations, suggesting some interference with the assay system.

In Vitro Transcellular Transport Assay. A transcellular transport assay (Table 3) was used to characterize a set of compounds which were identified by the P-gp screening assays (Table 2), suspected to be false negatives based on clinical data (Table 1, e.g., colchicine and digoxin), or were known to be P-gp substrates. In these experiments, the influence of P-gp on the apparent permeability of radiolabeled test compounds across cell monolayers was determined. Cells used for the assay were monolayers of wild-type LLC−PK1 cells, either untransfected or transfected with mouse (L-mdr1a) or human (L-MDR1) P-gp.[24,25] Compounds with a corrected transport ratio of greater than 1.5 were identified as P-gp substrates (Table 4). Species differences in P-gp transport were suggested by a greater than 2-fold

Table 3. In Vitro P-gp Direct Transport Assay. Transcellular Transport Ratios (basolateral-to-apical vs apical-to-basolateral transport) Using LLC-PK1, LLC-MDR1, and LLC-mdr1a Cells Are Shown[a]

	LLC-PK1	LLC-MDR1	LLC-mdr1a	LLC-MDR1 corrected	LLC-mdr1a corrected	P_e (nm/s)	substrate classification[b]
benzylpenicillin	1.0	1.0	1.1	1.0	1.1	255	−
cimetidine	6.7	3.3	6.5	0.5	1.0	136	−
colchicine	0.8	1.8	1.8	2.2	2.1	22	+
cyclosporin a	1.3	10.2	8.5	7.7	6.5	26	+++
dexamethasone	0.8	2.3	5.2	2.8	6.3	147	++/+++
digoxin	1.6	7.0	7.6	4.4	4.8	57	+++
enkephalin (dpdpe)	0.8	0.6	0.9	0.8	1.1	8	−
erythromycin	2.8	3.3	3.9	1.2	1.4	37	−
mibefradil	1.3	2.7	3.5	2.1	2.7	155	+/++
morphin	1.0	1.5	2.4	1.5	2.4	77	+
saquinavir	2.2	7.4	6.3	3.4	2.8	77	++
verapamil	1.1	6.1	6.6	5.7	6.2	216	+++
vinblastine	3.0	5.2	4.3	1.7	1.4	73	+/−

[a] Corrected transcellular transport ratios were calculated as the transport ratio obtained in either MDR1 or mdr1a cells, divided by the tranport ratio obtained in parental LLC-PK1 cells. Values are means of three to seven independent experiments ($n = 2$), each experiment performed in triplicate. Permeability coefficients (P_e) were obtained in LLC-PK1 cells in b-to-a direction. [b] Substrate classification for MDR1 and/or mdr1a: corrected transport ratio <1.5 (−), 1.5−2.5 (+), 2.6−3.4 (++), >3.5 (+++).

Table 4. Summary of in Vitro P-gp Screening Assays

assay	substrate concentration, μM[a]	activity measured	analytical requirements	throughput	criteria for "+" activity	ref
rhodamine assay or calcein-AM assay	≤50	inhibition	fluorescence	high	> 20% inhibition	
rhodamine assay or calcein-AM assay	0.1−50	inhibition	fluorescence	medium	IC_{50} < 30 μM	
ATPase assay	20	ATPase activity	absorbance	high	>1.2 nmol/mg/min	
ATPase assay	0.1−20	ATPase activity	absorbance	medium	>1.2 nmol/mg/min	
transport assay	≤20	transport	LC/MS/MS or radioactivity	medium to low	>1.5	44
in vivo (P-gp knock-out mice)		brain penetration	LC/MS/MS	low	K_p(brain,KO)/K_p(brain,WT) > 1	52

[a] May be adjusted according to expected systemic exposure in vivo.

Table 5. Recommendations on the Use of P-gp Assays during Different Stages of Drug Discovery and Drug Development

stage	throughput	analytical requirements	strategy
discovery (lead identification)	high	none	screening at ≤50 μM, rhodamine or calcein-AM assay
discovery (lead optimization)	high	none	screening at 0.1−50 μM, rhodamine or calcein-AM assay (if positive at 50 μM)
	high/medium	none and LC/MS/MS or radiolabeled compound	ATPase assay at 20 μM or 0.1−20 μM direct transport assay
development (clinical candiates)	low	LC/MS/MS or radiolabeled compound	in vivo PK (drug-drug interaction studies) P-gp knock-out mouse

difference in corrected transport ratios. Dexamethasone shows a species deference because it is a better substrate for mdr1a than for MDR1.

The transcellular transport assay suggests that cimetidine, enkephalin, and erythromycin are not substrates for P-gp. These compounds were shown to be negative in all P-gp inhibition assays, and exhibited low ATPase activity (Table 2). In addition, enkephalin was suggested to be a P-gp substrate in vivo because modulation of P-gp activity enhanced its antinociception.[27] An explanation for this apparent lack of transport is that low to moderate passive permeability is required for the manifestation of P-gp effects in transport experiments.[62] Compounds with high intrinsic permeability may pass through the cell monolayer faster than P-gp is able to remove them. This was suggested for midazolam and nifedipine,[44] where a fast transmembrane movement rate may overcome P-gp mediated efflux.[34,45] However, some compounds with very low intrinsic permeability were positive in ATPase assay.

Colchicine, morphine, and vinblastine exhibited a low transport ratio (1.5−2.4). Although colchicine was not identified as a substrate for the mouse homologue mdr1a, the results obtained for mdr1a were similar to those for MDR1. Mibefradile and saquinavir were better P-gp substrates (transport ratio 2.6 and 3.4). Dexamethasone, digoxin, cyclosporine A, and verapamil exhibited the highest transport ratio (>3.5). Digoxin and dexamethasone were not identified by inhibition assays despite their high transport rates by P-gp. The high transport rate by P-gp and low interaction potential may be explained kinetically by low affinity toward P-gp (high K_M) in combination with high maximal transport rate (high V_{max}). Thus, at very high concentrations of digoxin or dexamethasone, inhibition of P-gp might be observed.

Screening Strategies for Drug Discovery. It is difficult to classify test compounds as inhibitors, transported substrates, or nontransported substrates by a single assay because different experemental systems and test conditions produce different classification. It is important to note that P-gp does not influence the pharmacokinetics of P-gp inhibitors or nontransported

substrates. However, these compounds may modify the pharmacokinetics of coadministered P-gp substrates.

A clear strategy should be defined and followed in an early phase of drug discovery to identify potential inhibitors and substrates of P-gp. For screening in early stages of drug discovery, only the indirect fluorescence indicator screening assays are suggested. The risk of false negative results in these assays may be minimized by a more detailed assay procedure, i.e., by establishing a dose−response profile of the test compound, although throughput would be reduced. Cellular systems expressing P-gp of the species of interest should be used because of possible species differences. With respect to rodent P-gp, both isoenzymes, mdr1a and mdr1b, should be considered. These assays identify compounds that interact with P-gp, but they cannot distinguish substrates from inhibitors.

Inhibition and transport have to be regarded as distinct properties of the inhibitor and/or substrate of P-gp. Thus the fluorescence indicator assay should be combined with a methodology which allows for deciphering of the substrates from inhibitors of P-gp. Under certain circumstances, such as where Caco-2 permeability is low or oral bioavailablity is considerably lower than the theoretically achievable, or when brain penetration is lower than expected, a direct P-gp transport assay may be warranted, even if indirect assays were negative.

The ATPase assay at one substrate concentration predicted the results from more time-consuming in vitro transport assays, with the exception of digoxin and colchicine. This procedure allows more efficient screening for substrates (i.e., compounds classified as "+ + +" or "+ − −" in Table 2) or inhibitors of P-gp (i.e., compounds classified as "− + +").

In summary, the P-gp screening strategy outlined here has allowed us to classify structurally diverse compounds. The screening assays used in this study, as summarized in Table 4, do not require compound-specific analytical support and provide a high-throughput methodology for identification of inhibitors and substrates of P-gp. A large number of structurally diverse compounds allowed us to evaluate the advantages and disadvantages of the assays. We suggest that indirect fluorescence indicator assays (rhodamine or calcein-AM) should be used as the primary screen followed by an ATPase or transcellular transport assay to distinguish substrates and inhibitors as outlined in Table 5. The results from the screening assays should be confirmed at later stages of the drug development process by a direct transport assay, followed by in vivo experiments (e.g., P-gp knock-out mice).

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