Radioligand-Binding Assay Employing P-Glycoprotein-Overexpressing Cells: Testing Drug Affinities to the Secretory Intestinal Multidrug Transporter

Stephan Döppenschmitt,[1,2] Hildegard Spahn-Langguth,[1] Carl Gunnar Regårdh,[2] and Peter Langguth[2,3]

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Purpose. To develop a rapid and reliable system for affinity determination of conventional as well as newly synthesized compounds to P-gp.
Methods. The principles of radioligand-binding assay were adapted to the human intestinal P-gp. Acceptor protein was obtained from the human carcinoma cell line Caco-2, where overexpression of P-gp was induced by growing cells in the presence of the cytostatic drug vinblastine. ^{3}H-Verapamil was chosen as radioligand.
Results. The saturability and specificity of ^{3}H-verapamil as the radioligand for the binding to P-gp was demonstrated. From concentration dependence of displacement of the radioligand by various non-labeled ligands for P-gp, affinity constants to P-gp binding sites were calculated. The binding results obtained were in agreement with those published earlier where influx and efflux experiments with cell monolayers had been conducted in order to functionally characterize the P-gp -drug interaction.
Conclusions. A radioligand-binding assay on the basis of P-gp overexpressing Caco-2 cells has been developed. The method might be suitable for high-throughput screening of drug interaction with human P-gp. It will allow modeling of the interaction of drugs with the human multidrug transporter and has also the potential to serve as a high-throughput screening tool to detect compounds prone to P-gp mediated intestinal secretion and potential P-gp related drug/drug interactions in drug discovery and early development.

KEY WORDS: P-glycoprotein; binding affinity; radioligand-binding assay; ^{3}H-verapamil; drug secretion; drug efflux.

INTRODUCTION

For perorally administered drugs, the passage of the intestinal epithelium is, in addition to metabolism, one of the major hindrances on their way to the systemic circulation. The ability of a xenobiotic to cross the lipophilic barrier of the gut wall is, in the absence of a carrier system, determined by the physicochemical properties of the compound, i.e. its lipophilicity, its molecular weight, the ability to form ionized forms and hydrogen bonds. In addition to passive membrane permeation, intestinal metabolism and carrier-mediated transport mechanisms have been identified as factors, that may complicate the absorption of drugs after peroral administration. Among the carrier-mediated transport systems, an ATP-dependent exsorptive system has been discovered and identified (1,2) as the multidrug resistance protein P-glycoprotein (P-gp). This ABC (ATP-binding cassette)-family protein is located in the apical membrane of the enterocyte and mediates luminally-directed transport. Due to its saturability and influence on intestinal permeability, it may lead to dose-dependent drug absorption, discontinuous absorption profiles, prolongation in absorption times or to an overall low systemic availability, when secretion in the proximal GI-tract is not being compensated by absorption in the lower regions. An example is given by the β-adrenoceptor antagonist talinolol which has been identified showing typical P-gp modulated pharmacokinetic behaviour *in vitro* as well as *in vivo* (3,4).

It has been further pointed out, that due to the saturability of intestinal secretion, the impact of this counter-absorptive process might be of clinical relevance particularly for highly potent drugs administered in low doses (5). In this case a reliable assay for the determination of drug affinities to P-gp might be a valuable tool for the prediction of complex absorption profiles in early drug development. Such an assay system may likewise be of interest for the prediction of systemic drug-drug interactions, based on the competitive displacement between two or more drugs at P-gp binding sites.

Different approaches for the quantification of a compound's interaction with P-gp have been proposed, e.g. competition for cellular extrusion of fluorescent P-gp substrates (6) or quantification of the cellular ATP-ase activity (7,8). Most of theses methods, however, have drawbacks, that make proper interpretation of the data difficult. For the ATP-ase assay, it has been pointed out, that due to the use of membrane fractions of P-gp expressing cells as assay preparations the results may be biased due to changes in the lipid environment of the P-gp. This has been shown to influence the substrate recognition properties of P-gp (9). On the other hand, assays on the basis of intact cells (e.g. dye extrusion tests) usually result in mixed parameters reflecting drug membrane permeability and its affinity to P-gp. Thus a supplementary or alternative assay system is needed in order to quantitate drug affinity to P-gp. The radioligand-binding assay has been known as a valuable tool for the quantification of drug-receptor interactions, for instance of β-adrenoceptor antagonists (e.g. 10) or benzodiazepines (11). Drug-binding site interaction with this system is quantified by measuring the concentration-dependence of the displacement of a radiolabeled ligand specifically bound at the receptor or transporter (25).

An appropriate preparation with a high content of P-gp was developed using Caco-2 cells, a well established and stably P-gp expressing cell system. For the optimization of the ratio specific binding: non-specific binding, the expression of P-gp was induced by culturing cells in the presence of the cytotoxic substrate vinblastine (12). Prior to performing binding experiments, the cells were permeabilized, so that a rapid equilibrium between intracellular and extracellular concentrations of radioligand and competitor was achieved. This was by an—as opposed to methanol poration—mild poration method using lysolecithine, which is employed in molecular biology for transfection purposes (13).

[1] School of Pharmacy, Martin-Luther-Universität Halle Wittenberg, Wolfgang-Langenbeck-Strasse 4, D-06120 Halle (Saale), Germany.
[2] ASTRA HÄSSLE AB, PKDM, S-431 83 Mölndal, Sweden.
[3] To whom correspondence should be addressed. (e-mail: peter.langguth@hassle.se.astra.com)

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MATERIALS AND METHODS

Cell Culture

Dulbecco's Modified Eagle Medium, fetal calf serum (FCS), L-glutamine 200 mM, penicillin / streptomycin (10000 U/ml, 10000 μg/ml), Trypsin / EDTA, MEM non-essential amino acids (NEAA) and Hanks Balanced Salt Solution (HBSS) were from Life Technologies, Paisley, UK. Vinblastine sulfate and trypan blue solution (0.4%) were purchased from Sigma, Malmö Sweden. Transwell^R cell culture inserts used for transport experiments (24 mm, 0.4 μm pore size, polycarbonate membrane) and all other cell culture materials were from Costar, Cambridge, USA. The Caco-2 cells used were obtained from American Type Culture Collection (ATCC), Rockville, USA (passage 36) and J. Biber, University of Zürich, Switzerland (passages 74, 92).

Substances

^{3}H-verapamil (84 Ci/mmol) was from NEN, Boston, USA. Lysolecithine, adenosine triphosphate (ATP), creatine phosphokinase, creatine phosphate, verapamil, vinblastine, propranolol, indomethacin, tetraethylammonium (TEA), N^1-methyl-nicotinamide (NMN), caffeine, baclofen, phenobarbital, Pindolol, ouabain, quinin, quinidine, vincristine, acetaminophen, atenolol, chlorpromazine, cimetidine, doxorubicin, 5-fluorouracil and probenecid were obtained from Sigma, Malmö, Sweden, Morpholino ethane sulfonic acid (MES) was from Fluka, Gothenburg, rhodamine 123 (R123), calcein and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) from Molecular Probes, Leiden, Netherlands, MRK-16 mAb from Kamiya, Thousand Oaks, CA, USA and C219 mAb against P-gp from Centocor, Malvern, USA as well as the monoclonal antibody against MRP, QCRL-1. Talinolol was a gift from Arzneimittelwerk Dresden, Radebeul, Germany, metoprolol and ketoconazole from Astra Hässle, Mölndal, Sweden. 2′-deoxy-tubercidine (d-TUB) was purchased from TIB MolBiol, Berlin, Germany, testosterone from Steraloids, Wilton, USA, and celiprolol was a gift from Upjohn, Heppenheim, Germany. Floxuridin and tranylcypromine were gifts from D. R. Port, DKFZ Heidelberg, Germany and Procter and Gamble, Weiterstadt, Germany, respectively. The scintillation fluid Optiphase 'Highsafe' 3 was purchased from Wallac, Loughborough, UK. All other compounds and reagents used were from Sigma, Malmö Sweden.

Equipment

The MultiScreen 96-well plate assay system was from Millipore, Eschborn, Germany. The MultiScreen 96-well plates with Durapore membrane of 0.22 μm pore size and the MultiScreen 96-well plate punch tips were purchased from Millipore, Malmö, Sweden. Liquid scintillation was determined by a WinSpectral 1414 counter, Wallac, Turku, Finland.

Preparations of P-Glycoprotein

P-gp expression was induced in Caco-2 cell lines at different passage numbers (for comparison), starting with passages 36, 74 and 92 of commonly cultured Caco-2 cells. The cells were grown in 225 cm^2 flasks at 37°C in a 5% CO_2 atmosphere using DMEM containing 10 nM vinblastine, 16.5% FCS, 1% NEAA, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% L-glutamine. Cells were seeded at an initial density of 0.8–1 × 10^6 cells per flask and medium was changed every other day. Monolayers were trypsinized at 90–95% confluence and the cell suspension then used for radioreceptor-binding studies. Induced cell lines were characterized with respect to microscopic appearance, their ability to form monolayers and the transepithelial resistance of monolayers grown on Transwell^R inserts. To permeabilize cell membranes for binding studies, Caco-2 cells were suspended in lysolecithine-solution (0.01% [w:v] in HBSS with 10 mM MES, pH 7.0). Effectiveness of poration was checked by trypan blue exclusion test.

Radioligand-Binding Assay

Binding properties to P-gp were evaluated for ^{3}H-verapamil as the radioligand. Incubations were performed at 37°C under mild shaking conditions (Incubator S.I. 60, Stuart Scientific, UK) in HBSS solution containing 10 mM MES at pH 7.0 with 50 μl of P-gp preparation (1.25 10^6 cells per ml) in a total volume of 250 μl. The incubation medium was supplemented with ATP (1 mM) and an enzymatic system for the regeneration of ATP from ADP (ATP-system), containing magnesium chloride (10 mM), creatine phosphate (10 mM) and creatine kinase (100 μm/ml) (14). Filters were pre-washed with 100 μl of HBSS containing 10 mM MES at pH 7.0 (no additives) and the incubation was stopped after 30 min by vacuum filtration. This incubation period was chosen since kinetic experiments (10 μM concentration of verapamil containing 2.4 nM of ^{3}H-labeled compound) had demonstrated a rapid onset for the association as well as for the dissociation of verapamil with the P-gp preparation, reaching their equilibria within 10 to 15 min. Filters were washed twice with 100 μl ice-cold HBSS containing MES pH 7.0 (no additives), incubated with 5 ml of scintillation fluid for 12–16 h at RT, and total radioactivity on the filters was counted by liquid scintillation counting (Wallac WinSpectral 1414 Liquid Scintillation System, Upplands Väsby, Sweden).

Saturation experiments: The saturable binding of verapamil to the intestinal P-gp was characterized with respect to maximal binding capacity (B_{max}) and concentration of verapamil necessary for half-maximal binding (K_d) in the concentration range from 0 to 13 μM. For determination of total binding, verapamil solutions of 12 different concentrations in this range, obtained by spiking solutions of non-labeled verapamil with nanomolar concentrations of radiolabeled drug, were incubated in duplicate with the P-gp preparation in presence of the ATP-system. Non-specific binding was determined for every second concentration used for the determination of the total binding by adding 1 mM rhodamine 123 (R123) for saturation of specific binding.

Competition experiments: In order to evaluate the affinity of non-labeled ligands for P-gp, incubations were performed in duplicate with a fixed concentration of 100 nM verapamil (containing labeled verapamil at 2.4 nM concentration) and 16 different concentrations of competitor between 0 and 35 mM.

Specificity of Radioligand Binding to P-gp

To test the specificity of radioligand binding to P-gp, competition experiments with two monoclonal antibodies against

different epitopes of P-gp, MRK-16 (Kamiya, Thousand Oaks, CA, USA) and C219 (Centocor, Malvern, USA) were performed. Further rhodamine 123 (0–0.8 mM), verapamil (0–0.8 mM), vinblastine (0–0.8 mM), talinolol (0–20 mM), metoprolol (0–4.0 mM), propranolol (0–10 mM), calcein (0–1.0 mM), 2′,7′-bis (2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (5.0 mM), indomethacin (1.0 mM), monoclonal antibody against MRP, QCRL-1 (0.5 μg/ml), tetraethylammonium (TEA) (5.0/50.0 mM), N^1-methyl-nicotinamide (NMN) (1.0/5.0 mM), probenecid (1.0 mM), 2′-deoxy-tubercidine (1.0/5.0 mM), testosterone (2 mM), celiprolol (0–35 mM) were evaluated for their competition with the radioligand. Furthermore, the concentration dependent competition between talinolol and the radioligand was tested in the presence of fixed concentrations of ligands for other potential binding sites (testosterone, 2 mM, calcein, 0.5 mM, probenecid, 1.0 mM, d-TUB, 1.0 mM, NMN, 1.0 mM). DMSO (Sigma) or ethanol (Kemethyl, Haninge, Sweden) was added to the solutions when necessary (concentration up to 1%), which did not affect the binding of the radioligand as shown in control experiments.

Reproducibility

To establish reproducibility, competition experiments were carried out repeatedly on different days for the model compounds verapamil and talinolol.

Data Analysis

Saturation experiments: For the determination of specific radioligand binding, non-specific binding was determined by linear regression analysis and subtracted from total binding. To the specific binding data, a Hill-equation for two-affinity sites was fitted [23] according to:

$$B_{observed,specific} = B_{max,specific} \cdot \left(\left(\frac{f_1 \cdot RL^{n_1}}{K_{d1}{}^{n_1} + RL^{n_1}}\right) + \left(\frac{f_2 \cdot RL^{n_2}}{K_{d2}{}^{n_2} + RL^{n_2}}\right)\right)$$

$B_{observed,\ specific}$	bound radioactivity observed
$B_{max,\ specific}$	maximal bound radioactivity
RL	concentration of radioligand
f_1	fraction of first class of binding sites
n_1	Hill-coefficient for first class of binding sites
K_{d1}	dissociation constant for first class of binding sites
f_2	fraction of second class of binding sites
n_2	Hill-coefficient for second class of binding sites
K_{d2}	dissociation constant for second class of binding sites.

For verapamil, the Hill coefficients were 1.2 and 5.9 for the first and the second binding site, respectively, f_1 was fixed to 0.5 for parameter optimization according to the height of the first plateau of the saturation curve, and $f_2 = 1 - f_1$.

Competition experiments: IC_{50}-values were determined by fitting the equation:

$$B_{observed} = B_{i\to\infty} + (B_{i=0} - B_{i\to\infty}) \cdot \left(\frac{RL^n}{RL^n + IC_{50}{}^n}\right)$$

to the obtained data. Alternatively, a two-affinity model was fitted without data transformation [24], in which the Hill coefficients were set to 1:

$$B_{observed} = B_{i\to\infty} + (B_{i=0} - B_{i\to\infty}) \cdot \left(\left(\frac{f_1 \cdot RL}{RL + K_{d1} \cdot \left(1 + \frac{i}{K_{i1}}\right)}\right) + \left(\frac{f_2 \cdot RL}{RL + K_{d2} \cdot \left(1 + \frac{i}{K_{i2}}\right)}\right)\right)$$

$B_{observed}$	bound radioactivity observed
RL	concentration of radioligand
i	concentration of competitive inhibitor
$B_{i\to\infty}$	bound radioligand in presence of high concentration of inhibitor
$B_{i=0}$	bound radioactivity in absence of inhibitor
f_1	fraction of first class of binding sites
f_2	fraction of second class of binding sites
K_{d1}	dissociation constant of RL for first class of binding sites
K_{d2}	dissociation constant of RL for second class of binding sites
K_{i1}	dissociation constant of inhibitor for first class of binding sites
K_{i2}	dissociation constant of inhibitor for second class of binding sites
n	Hill coefficient

For nonlinear regression analysis SigmaPlot 2.01 (Jandel Scientific, USA) and for all other calculations Excel 5.0, (Microsoft, USA) was used.

RESULTS

The induced Caco-2 cells from different passage numbers did not show any morphologic differences when compared with the non-induced cells with respect to microscopic appearance, formation of tight monolayers and transepithelial resistance. The permeabilization process using lysolecithine, to allow free access of radioligand and competitor to the intracellular compartment, resulted in more than 99% porated cells as determined by the trypan blue dye exclusion method directly after application of the lysolecithine and was not reversible under the assay conditions for at least one hour.

The binding of the radioligand ^{3}H-verapamil was characterized with respect to saturability and non-specific binding (Fig. 1). Due to the apparent two-step course of the saturation curve, a two-affinity model was used for the evaluation of association and competition experiments. This model was statistically proven to be superior over the single affinity model by comparison of the sum of squares of the respective fitting result for each model by means of an F-test (F-statistic 7.5, $p < 0.05$, DF n = 2, DF n = 5). However, for better comparison to affinity-data previously published by other groups, e.g. from e.g. transport experiments or ATP-ase assay, where only a single affinity model has been used, IC_{50} values were also calculated. The affinity constants (K_d-values) found for ^{3}H-verapamil in saturation experiments (K_{d1} = 0.56 ± 0.13 μM, K_{d2} = 3.8 ± 1.8 μM) were reproduced in competition experiments (K_{i1} = 0.27 ± 0.32 μM, K_{i2} = 3.6 ± 1.3 μM) using cold verapamil as displacer. Competition experiments with monoclonal antibodies against P-gp, MRK16

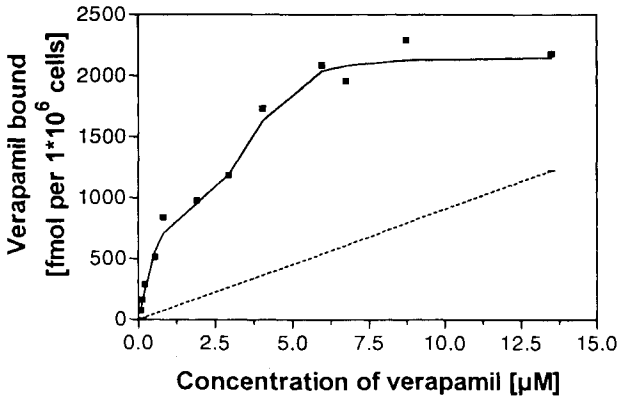

Fig. 1. Typical binding curve demonstrating the association of verapamil with the P-glycoprotein overexpressing Caco-2 cells. The curves represent specific (■ mean, n = 2 for each concentration) and non-specific binding (------) of verapamil as well as the fit obtained by the two-affinity model (———). Affinity constants were K_{d1} = 0.52 µM, K_{d2} = 3.8 µM, respectively.

and C219 with ^{3}H-verapamil showed a concentration-dependent displacement of the radioligand, however, concentrations of the antibodies achievable in the assay were not sufficient for full displacement of radioligands (Table I), whereas the fluorescent dye R 123, a specific substrate for P-gp but not for multidrug-resistance related protein (MRP) (15), displaced ^{3}H-verapamil down to non-specific binding levels. Experiments carried out to establish the contribution of other affinities than P-gp to the saturable binding, showed, that other possible binding sites were too low in abundance or affinity to contribute to the total binding to a relevant extent. The presence of CYP 3A4, MRP and the organic cation transporter, OCT, could be excluded by competition experiments with specific ligands for each transporter (Table I). Interestingly, TEA and NMN, commonly used substrates for the functional characterization of OCT, were able to displace the radioligand, but since high concentrations (50 mM) of TEA were able to displace ^{3}H-verapamil to baseline values, it was concluded, that TEA and NMN are probably not specific for OCT. The presence of OCT was excluded in this preparation due to the lack of displacement of the radioligand by probenecid and d-TUB. Both substrates have been shown previously—using Xenopus laevis oocytes expressing either P-gp or OCT—to be specific substrates for OCT, but not for P-gp (16,17). Competition experiments with the P-gp substrate talinolol in the presence of fixed concentrations of ligands for other binding-sites, such as CYP 3A4 (testosterone), MRP (calcein) or OCT (probenecid, d-Tub, NMN), were also carried out (Table IB). Merely in the case of NMN a significant impact on the IC_{50} of talinolol was detected, consistent with the findings in experiments described above, where NMN was shown to be a non-specific ligand for OCT. The binding of ^{3}H-verapamil to calcium channels was not entirely ruled out in the experiments performed here, however it is known, that verapamil affinity to Ca-channels in intestinal epithelial cells is characterized by a K_d-value of 100 µM [17], which is considerably higher than affinities determined for P-gp by this radioligand-binding assay.

The assay reproducibility was documented by repeated competition experiments for verapamil and talinolol representing a high (IC_{50} 0.8 µM, and a low affinity (IC_{50} 1.25 mM) ligand for P-gp (Fig. 2), respectively. The results demonstrate good reproducibility for competition experiments performed on different days, an important requirement for the comparability of the data obtained from different runs.

Affinities to P-gp using the RLB assay were determined for the MDR-modulator verapamil, the cytostatic drug vinblas-

Table IA. Results from Competition Experiments with R123 as High Affinity Ligand for P-gp, the P-gp Specific Monoclonal Antibodies MRK16 and C219, Calcein, Indomethacin, and BCECF as Ligands or Modulators for the Multidrug-Resistance Related Protein (MRP), Respectively, as Well as the Monoclonal Antibody Against MRP, QCRL-1

Substance	Concentration	^{3}H-Verapamil bound, % (means ± SD, n=3)
R123	0.1 mM	13.1 ± 0.8
	0.8 mM	7.1 ± 0.1
C219	0.75 mg/ml	66.5 ± 0.2
	1.125 mg/ml	56.7 ± 0.07
MRK16	10 µg/ml	80.6 ± 2.8
Calcein	1 mM	105.9 ± 7.5
Indomethacin	1 mM	93.8 ± 3.5
BCECF	0.5 mM	98.1 ± 9.6
QCRL-1	0.5 µg/ml	113.2 ± 3.3
Probenecid	5 mM	99.3 ± 5.1
TEA	5 mM	27.1 ± 2.5
	50 mM	20.7 ± 0.6
NMN	1 mM	72.5 ± 12.1
	5 mM	70.9 ± 2.8
d-TUB	1 mM	92.3 ± 2.1
	5 mM	96.2 ± 5.3

Note: Furthermore ligands or modulators for the organic cation transporter (OCT) probenecid, tetraethylammonium (TEA), N-methyl nicotinamide (NMN) and 2′-deoxy-tubercidine (d-TUB) were included in competition experiments. ^{3}H-verapamil bound to the P-gp preparation in presence of the respective concentration of competitor as percentage of the bound radioactivity in its absence (means ± SD, n = 3).

Table IB. Results from Competition Experiments with the P-gp Ligand Talinolol in the Absence or Presence of Competitors for Other Potential Binding Sites

Second competitor	Concentration (mM)	IC_{50} Talinolol, µM (means ± SD, n = 3)
None	--	1064 ± 105
Testosterone	2	1182 ± 170
Calcein	0.5	1244 ± 183
Probenecid	1.0	983 ± 127
d-TUB	1.0	1161 ± 110
NMN	1.0	844 ± 92

Note: ^{3}H-verapamil was used as the radioligand. Testosterone was tested as ligand for cytochrome P450 3A4, calcein as ligand for MRP and probenecid, 2′-deoxytubercidine (d-TUB) and N-methyl-nicotinamid (NMN) as ligands for the OCT, respectively. Mean IC_{50}-values for talinolol ± SD, n = 3 are given.

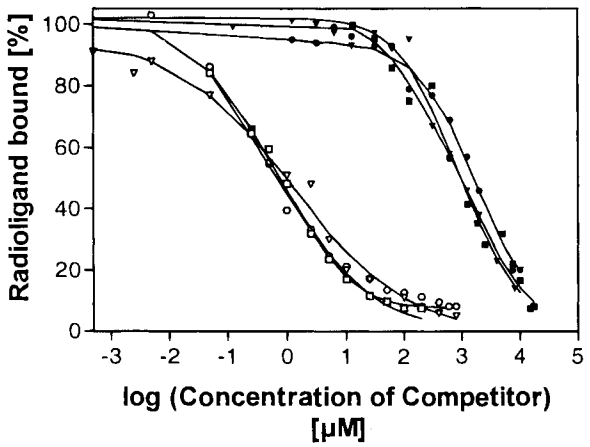

Fig. 2. Repeated competition experiments on three different days for verapamil (open symbols) and talinolol (filled symbols). Total ^{3}H-verapamil bound as a function of concentration of competitor (16 different concentrations for each day and compound, means of duplicate determinations) and fit as obtained according to the two-affinity model.

tine, for a group of β-adrenoceptor antagonists, as well as for R123, a widely used fluorescent dye for functional characterization of P-gp by dye extrusion test (Table II). The IC_{50}-values for these compounds, determined by the RLB assay were in good agreement to values described in the literature, obtained either by dye extrusion tests, for verapamil (6), or from transport experiments using Caco-2 monolayers for celiprolol (18) or vinblastine (19).

DISCUSSION

The affinity to secretory P-gp in the gastrointestinal tract has been shown to be a potential source of drug-drug or drug-food interactions with respect to the extent and velocity of absorption after oral administration (12). This might be particularly true for highly potent and thus low-dosed drugs currently under development. Therefore, in drug discovery and early drug development, knowledge on the affinity to such transport systems may be valuable information for the prediction of possible complications in the pharmacokinetics of new drug candidates.

Other experimental methods for the determination of drug affinities to P-gp have been described previously (5). However, as pointed out recently (5), *in vivo* perfusion and *ex vivo* transport studies using animal intestinal segments and mucosal sheets seem less useful due to the limited throughput and the inherent variability of such methods. *In vitro* transport studies using Caco-2 monolayers have also been applied (3), however this approach requires considerable effort in demonstrating direction specificity, concentration dependence as well as competitive inhibition studies, thus rendering it time consuming and far from adequate for drug screening programs. Various functional

Table II. Results from Competition Experiments to ^{3}H-verapamil as Radioligand, K_i-Values for the Two-Affinity Model as Well as IC_{50}-Values

	Two-affinity model[a]			One-affinity model[b]		
Compound	K_{i1} [μM]	K_{i2} [μM]	f1	IC_{50} [μM]	n	From literature [μM] (Ref.)
Acetaminophen	1)	-	-	-	-	--
Atenolol	1)	-	-	-	-	--
Baclofen	1)	-	-	-	-	--
Caffeine	1)	-	-	-	-	--
Celiprolol	40	2600	0.5	730	0.6	1000 (18)
Chlorpromazine	0.6	35	0.8	1.2	0.8	--
Cimetidine	2)	-	-	6500	0.9	--
Doxorubicin	2)	-	-	990	1.0	--
5-Fluorouracil	1)	-	-	-	-	--
Floxuridine	1)	-	-	-	-	--
Haloperidol	0.2	15	0.3	5.3	0.4	--
Ketoconazole	1.2	44	0.5	13	0.7	--
Metoprolol	200	1750	0.2	1300	0.8	--
Phenobarbital	1)	-	-	-	-	--
Pindolol	2)	-	-	1950	0.7	--
Propranolol	48	790	0.5	350	0.8	--
Ouabain	1)	-	-	-	-	--
Quinidine	2.6	485	0.2	340	0.6	--
Quinine	12	620	0.2	430	0.6	--
Rhodamine 123	0.1	3.3	0.7	0.5	0.6	--
Talinolol	140	1800	0.3	830	0.8	--
Tranylcypromine	2)	-	-	14000	0.5	--
Verapamil	0.3	3.6	0.4	1.5	0.5	1.0 (6)
Vinblastine	0.1	48	0.4	34	0.5	19 (9)
Vincristine	0.5	150	0.2	86	0.5	--

[a] For the two-affinity model the HILL-coefficients were fixed to 1 for both affinities.
[b] For the one-affinity model the HILL-coefficient (n) was evaluated by non-linear regression. 1) No competition to verapamil detected up to a concentration of 4 mM. 2) Two-affinity model could not be evaluated due to solubility-limitation of the compound in competition experiments.

P-gp assays have also been described where the extrusion of various dyes, e.g. R123 or calcein AM, as a function of the concentration of P-gp modulating compound is measured. These methods, however, show drawbacks due to the fluorescence dependence on the intracellular distribution of the dye, the intracellular Ca-concentration or the pH (21,22). Furthermore, the apparent affinity of a compound determined in such an assay system is always a mixed parameter of its affinity to P-gp and cell membrane permeability.

In contrast, radioligand-binding assays are well established screening methods for the identification of ligands for pharmacological receptors like β-adrenoceptor antagonists or benzodiazepines (e.g. 10). The technique allows the determination of the affinity for any unlabeled compound by measuring the concentration-dependence of the displacement of a radiolabeled ligand (radioligand) from the binding site of interest. The interaction of pairs of ligands for P-gp is likely to be of a competitive nature (2), such that the radioligand-binding assay to quantify drug—P-gp interaction and to obtain a rapid and reliable method for direct determination of affinity constants to P-gp is achievable. By using 96-well incubation and filtration equipment, the assay is suitable for high throughput in drug screening programs.

CONCLUSIONS

The results obtained for selected compounds with this assay were in good agreement with the limited number of results available by alternative methodologies, e.g. transport studies across Caco-2 cells and measurement of ATP-ase activity in the presence of increasing amounts of ligands for P-gp. It should also not be overlooked, however, that the determination of ATP-ase activity did result in rather complex activation and inhibition profiles (6,8). Also, some ligands for P-gp were shown to be able to modulate ATP-ase activity only in the presence of verapamil, while others stimulated ATP turn-over without further addition of drugs (20), making results occasionally difficult to interpret.

In conclusion, the radioligand-binding assay for P-gp introduced here provides, for the first time, the opportunity for simple and rapid determination of binding of compounds to human P-gp. In future work, affinity constants, together with diffusional membrane permeabilities, will represent valuable experimental information for modeling the impact of P-gp on the intestinal absorption of new drugs. This should lead to a better understanding of the *in vivo* relevance of exsorption mechanisms in pharmacokinetics.

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