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Identification of P-glycoprotein substrates and inhibitors among psychoactive compounds – implications for pharmacokinetics of selected substrates

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Abstract

The pharmacokinetics of antipsychotic drugs has become an integral part in understanding their pharmacodynamic activity and clinical effects. In addition to metabolism aspects, carrier-mediated transport, particularly secretion by ABC transporters, has been discussed as potentially relevant for this group of therapeutics. In this study, the psychoactive compounds perphenazine, flupentixol, domperidone, desmethyl clozapine, haloperidol, fluphenazine, fluvoxamine, olanzapine, levomepromazine, perazine, desmethyl perazine, clozapine, quetiapine and amisulpride were characterized in terms of P-glycoprotein (P-gp) affinity and transport. Experimental methods involved a radioligand displacement assay with [^{3}H]talinolol as radioligand and transport – as well as transport inhibition – studies of the P-gp substrate [^{3}H]talinolol across Caco-2 cell monolayers. In addition, the physicochemical descriptors log P and Δlog P were determined to test potential correlations between transporter affinity and lipophilicity parameters. All of the tested antipsychotics showed affinity to P-gp albeit their IC50 values (concentration of competitor that displaced 50% of the bound radioligand) differed by a factor exceeding 1000, when compared using the transport inhibition assay. From the group of P-gp substrates, amisulpride and fluphenazine were selected for in-vivo drug–drug interaction studies in rats to demonstrate the in-vivo relevance of the in-vitro findings. Compounds were administered by intraperitoneal injection either alone or in combination with 50 mg kg^{-1} ciclosporin. The concentration versus time profiles for both drugs were followed in serum as well as in brain tissues. Significant differences between the treatments with the antipsychotic alone versus the combination of antipsychotic with ciclosporin were found for amisulpride. The distribution of amisulpride to the brain was increased and systemic serum levels were likewise increased indicating decreased systemic clearance for the combination regimen. For fluphenazine, systemic levels with and without co-administraton of ciclosporin were comparable while higher brain-to-serum concentration ratios were found after co-administration of ciclosporin. The findings are explained on the basis of the limited contribution of P-gp-mediated transport to the elimination of fluphenazine and to a direct effect with respect to its distribution into the brain.

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Introduction

Pharmacokinetic drug–drug interactions (e.g., with selective serotonin re-uptake inhibitors (SSRIs)), as well as polymorphisms of cytochrome P450 enzymes, which interfere with drug clearance, may affect the safety and efficacy of antipsychotic drugs (Taylor 1995; Bertilsson & Dahl 1996; Nemeroff et al 1996).

The importance of carrier-mediated transport for absorption and disposition of drugs and xenobiotics has been described (e.g., Tsuji & Tamai 1996; Suzuki et al 1997). Changes in ADME parameters may occur as a result of inhibition or induction of one or several membrane transporters. For the blood–brain barrier, the role of exsorptive transporters has been pointed out (Kim 2003; Zong & Pollack 2003) and it has been shown that these transporters contribute to the limited access of their substrates to brain tissues. Among these exsorptive transporters, the MDR1 gene product P-glycoprotein

plays a dominating role, since it accepts a wide spectrum of chemically diverse compounds, most of which are relatively hydrophobic and carry a positive charge at physiological pH. Tsuruo et al (1981) first reported that verapamil and the neuroleptic drug trifluperazine potentiated the antiproliferative activity and increased cellular accumulation of vincristine in an MDR murine leukaemic cell line. Since this original observation, many compounds have been shown to antagonize MDR in a variety of in-vitro and in-vivo models when co-administered with chemotherapeutic agents to which the cells are resistant. One model compound, which has extensively been investigated in in-situ- and in-vivo studies in rats, is the β-adrenoceptor blocking agent talinolol (Spahn-Langguth et al 1998; Hanafy 2001).

In this study, 14 psychoactive compounds were characterized with respect to their P-glycoprotein inhibitory potency using an established radioligand binding assay and transport inhibition of the P-gp model substrate talinolol across Caco-2 cell monolayers. Additionally, the subset of compounds that were subject to direction-specific transport in the secretory direction across Caco-2 cell monolayers was studied. Finally, in-vivo pharmacokinetic interaction studies were carried out with selected P-gp substrates in rats, in which brain as well as plasma concentrations were measured with and without co-administration of ciclosporin as P-gp inhibitor. The in-vivo study was designed to demonstrate the relevance of the in-vitro findings.

Materials and Methods

Drugs

The following drugs were included in the study: perphenazine base, flupentixol dihydrochloride, domperidone base and desmethyl clozapine (all Sigma-Aldrich Chemie GmbH, Steinheim, Germany), haloperidol base, fluphenazine dihydrochloride (both RBI, Research Biochemical International, Natick, USA), olanzapine base (Eli-Lilly & Co, Indianapolis, USA), levomepromazine hydrochloride (Bayer, Wuppertal, Germany), perazine dimalonate, desmethyl perazine base (both Promonta, Hamburg, Germany), clozapine base (Sandoz Novartis Generics, Weil, Germany), fluvoxamine maleate (Solvay Pharma, Hannover, FRG), quetiapine base (AstraZeneca, London, UK) and amisulpride base (Sanofi-Synthelabo, Paris, France). Racemic talinolol was a gift from Arzneimittelwerk Dresden (Radebeul, Germany). [^{3}H]Talinolol was obtained from J. Kix (Volxheim, Germany). Vinblastine sulphate (Velbe) was from Lilly (Bad Homburg, Germany). Verapamil hydrochloride was obtained from ICN Biomedicals GmbH (Eschwege, Germany).

Cell culture media

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), non essential amino acids (NEAA), penicillin–streptomycin solution (PEST), trypsin–ethylenediaminetetraacetic acid (trypsin-EDTA), Dulbecco's modified phosphate-buffered saline (PBS) and Hank's balanced salt solution (HBSS) were obtained from Biochrom KG (Berlin, Germany). 2-Morpholino-ethanesulfonic acid monohydrate (MES) and sodium hydroxide were purchased from Merck (Darmstadt, Germany). D-Mannitol was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). [^{14}C]Mannitol (specific activity 53 mCi mmol^{-1}) was obtained from Biotrend (Köln, Germany). Scintillation fluid Rotiszint 22 was purchased from Carl Roth GmbH & Co (Karlsruhe, Germany). Water was purified and de-ionized by a Milli-Q water processing system (Millipore, Eschborn, Germany). All other chemicals and reagents were of analytical grade and were purchased from commercial sources.

HPLC equipment

The quantification of psychoactive compounds was performed by HPLC. The separation was carried out on a Lichrospher CN column, 5 μm, 125 × 3 mm, and on a Lichrospher CN column, 5 μm 250 × 4.6 mm, for the samples from the in-vitro and in-vivo studies, respectively. The system was equipped with an HPLC pump (Bischoff-pump, model 2250; Bischoff, Analysentechnik, GmbH, Leonberg, Germany), an autosampler (Sampling injector 231-401; Gilson medical electronics, Middleton, USA), a UV detector (SPD-10A UV/VIS detector; Shimadzu Corporation, Kyoto, Japan) and integration system (Kromasystem 2000; PC Kroma integration Pack, Kontron Instruments SRL, Milan, Italy).

Equipment for transport studies

The transport experiments were conducted using an incubator BE 500 (Memmert, Schwabach, Germany), a Bühler shaker KMA Vario (Johanna Otto GmbH, Hechingen, Germany), and Transwell 12-mm polycarbonate membrane inserts, 0.4-μm pore size, 1.13-cm^2 surface area (Transwell; Corning Costar, Bodenheim, Germany). Radioactivity was counted on a liquid scintillation counter LC 6000 (Beckman Coulter, Unterschleissheim, Germany). Scintillation vials (Mini vials A) were obtained from Carl Roth GmbH & Co (Karlsruhe, Germany).

Determination of log P, Δlog P and pKa

Log P values were calculated using the QMPR Plus software program (Simulations Plus, Lancaster, USA). Experimental determinations were carried out on a Sirius PCA 200 pKa and log P instrument (Sirius Analytical Instruments Ltd, Forest Row, East Sussex, UK) at 25 ± 0.5°C under an inert nitrogen gas atmosphere to exclude CO_2. Control 200TM and Refine 200TM (Version 5.2a) software was used to manage the transfer of data between the PC and the Sirius PCA 200 and for the calculation of pKa, log P and Δlog P, respectively. Due to the poor water solubility of several of the investigated compounds, pKa values were determined by a mixed water/co-solvent method. Semi-aqueous solutions (20 mL each, total of 2–3 determinations) containing 9 to 43% (w/w) methanol as co-solvent and 0.15 M KCl as

ionic strength adjuster, as well as 0.245–0.962 mM of the test substance, were acidified by 0.5 M HCl to an initial pH of 1.8. The solutions were titrated with 0.5 M KOH to a pH of 12. After each addition of titrant the pH was measured. The multi-titration curves thus obtained will produce different values of psKa(s), apparent ionization constants, in the presence of co-solvent, which were obtained from the difference Bjerrum plots. The difference Bjerrum curve is defined as the difference between two titration curves: one curve is that of a titration of an ionizable substance and the other that of a blank titration (ionic strength adjusted aqueous solution without test substance), performed under nearly identical conditions. The difference Bjerrum curve is the plot of the calculated average number of bound protons per molecule of the sample versus pH. When this number equals 0.5 (i.e., at 50% ionization), the pH value equals the apparent ionization constant. These values are then refined by a weighted non-linear least-squares procedure (Refine 200 program) to create a multiset, where the refined values are then extrapolated to zero co-solvent concentration to obtain the aqueous pKa values using the Yasuda-Shedlovsky equation (Avdeef et al 1999):

$$\mathrm{psKa} + \log[H_2O] = a/\varepsilon + b \quad (1)$$

where psKa is the apparent ionization constant in the presence of co-solvent, $\log[H_2O]$ is the logarithm of the molar concentration of water, ε is the dielectric constant of the water/co-solvent mixture and a and b are the slope and intercept of the regression line, respectively. The aqueous pKa values were obtained by plotting the psKa values and the logarithm of the molar concentration of water versus the reciprocal of the dielectric constant of the water/co-solvent mixture. pKa values for all investigated compounds were determined using ionic strength-adjusted water by the addition of 0.15 M KCl–methanol mixtures, except for haloperidol, where dimethyl sulfoxide was employed as co-solvent (30–43%, w/w). For levomepromazine hydrochloride, the pKa was determined in ionic strength-adjusted water by addition of 0.15 M KCl without addition of co-solvent. The pKa was determined according to the following equation (Albert & Seargent 1984):

$$\mathrm{pKa} = \mathrm{pH} + \log[BH]^+/[B] \quad (2)$$

HPLC analysis

Samples from in-vitro permeation studies were analysed by HPLC, modified from Härtter and Hiemke (1992), at ambient temperature using a mobile phase composed of acetonitrile–phosphate buffer pH 6.4 (50:50, v/v) at a flow rate of 1 mL min^{-1}. The phosphate buffer was prepared by dissolving 1.85 g of dipotassium hydrogen phosphate trihydrate in 1000 mL of distilled water and adjusting the pH by adding *o*-phosphoric acid 85%. The wavelength for UV absorbance was set at 214 nm. The sample injection volume was 150 μL.

Radioligand-binding assay (RBA)

All radioligand-binding experiments were performed using a cell harvester (Brandel Cell Harvester, M-24 R; Biomedical Research and Development Laboratories, Gaithersburg, USA), MicroWell plates (Nunc, Wiesbaden, Germany), GF/C filters pore size 1.2 μm (Dunn Labortechnik GmbH, Asbach, Germany) and a shaker (IKA MTS 2; IKA Werke, Staufen, Germany).

P-gp-over-expressing Caco-2 cells (Passage 64) were grown in 75-cm^2 tissue flasks in DMEM supplemented with 20% fetal bovine serum, 1% non-essential amino acids, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10 nM vinblastine sulfate in an atmosphere containing 5% CO_2 at 95% humidity. The cells were washed twice with phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} and were routinely subcultured once a week with trypsin-EDTA solution. Cells used for the RBA were suspended in HBSS buffered with 10 mM MES (pH 7.0) containing 0.01% (w/v) of l-α-lysophosphatidylcholine. The RBA was conducted in a 96-well plate with a total volume of 250 μL for each well. A 100-μL volume of the different concentrations of each test compound dissolved in HBSS buffered with 10 mM MES was given into each well. In addition, 25 μL of radioligand solution (talinolol 1 μM containing 20% [^{3}H]talinolol and 125 μL of the cell suspension were added. Following incubation at room temperature and shaking for 30 min (1200 rev min^{-1}), the solutions were filtered through glass fibre filters in a harvester and unbound radioligand was removed by purging the filters with ice-cold 0.9% sodium chloride. Thereafter the filters were dissolved in 4 mL scintillation fluid and counted in a liquid scintillation counter. The concentration of competitor that displaced 50% of the bound radioligand (IC50) was calculated from displacement curves using GraphPad Prism Ver. 3.0 (GraphPad Software, San Diego, USA) by non-linear regression analysis employing a sigmoidal concentration–response (variable slope) relationship:

$$Y = (\mathrm{Top} - \mathrm{Bottom})/(1 + 10^{(\log IC50 - X * \mathrm{Hill\ Slope})}) \quad (3)$$

where X is the logarithm of the concentration of test compound (competitor), Y is the percentage of specific bound radioactivity (% B/B_0), B is the specific bound radioactivity at different competitor concentrations, B_0 is the maximum bound radioactivity in buffer, log IC50 is the X-value when the response (percentage of specific bound radioactivity) is halfway between bottom and top, the Hill Slope describes the steepness of the curve, Top is the maximum value of the competition curve, and Bottom is the minimum value of the competition curve.

Transport studies

For transport experiments, P-gp-expressing Caco-2 cells were cultivated for 21–29 days on Transwell polycarbonate filters (area 1.13 cm^2, pore size 0.4 μm). The integrity of each monolayer was monitored by TEER (transepithelial electrical resistance) using a Millicell ERS

and chopstick electrodes. In addition, [^{14}C]mannitol was applied to the donor solution at the end of the experiment to reach a final concentration of 1 μCi mL^{-1}. Effective permeability coefficients (P_{eff}) for mannitol were between 9.3×10^{-8} and 1.05×10^{-6} cm s^{-1}. As transport medium, HBSS supplemented with 10 mM MES adjusted to pH 7.4 was used throughout all experiments. Each experiment was performed in triplicate at 37°C. Effective permeability coefficients were calculated from the slopes of cumulative amounts permeated versus time according to:

$$P_{eff} = (dc/dt \times V)/(A \times C_o) \quad (4)$$

where dc/dt is the steady-state change in concentration in the receiver chamber, V is the volume of the receiver chamber, A is the surface area of the monolayer, and C_o is the initial concentration of the compound in the donor compartment. Transport experiments of the antipsychotic compounds were conducted at 0.1 mM initial donor concentrations. Receiver samples were taken at 0, 30, 60, 90 and 120 min and the sample volume was replaced by blank transport buffer. Transport inhibition experiments were conducted with talinolol at 0.1 mM donor concentrations containing 1 μCi mL^{-1} [^{3}H]talinolol in the presence of the putative inhibitor compound.

Pharmacokinetic studies in rats

Male rats, 180–200 g, of the strain PVG/OlaHsd (PVG), were obtained from Harlan-Winkelmann (Borchen, Germany). The rats had free access to food and water and were maintained under a 12-h light–dark cycle, housed three per cage at 22°C and a relative humidity of 60%. All experiments with rats were conducted in accordance with the U.S. guide for the care and use of laboratory animals and approved by local authorities. The rats were treated either with amisulpride (50 mg kg^{-1}, i.p.) + ciclosporin (50 mg kg^{-1}, i.p.) or with amisulpride only (50 mg kg^{-1}, i.p.). In the case of fluphenazine, the dose was 1 mg kg^{-1}, i.p., with or without ciclosporin (50 mg kg^{-1}, i.p.). Truncal blood was collected following decapitation of rats at 1, 3, 6, 9, 15 and 24 h after amisulpride administration (0.5, 1, 3, 6, 12, 24 and 30 h after fluphenazine administration) and the brain was removed within 30 s and frozen in liquid nitrogen. Blood was allowed to clot and serum was obtained following centrifugation at 3000 *g* for 10 min. Serum and rat brain were stored at −20°C until analysis. For HPLC analysis serum samples were processed directly without further purification by a fully automated chromatographic method including on-line clean-up, isocratic liquid chromatography and UV spectrophotometric detection at 254 nm. Samples were injected onto a small clean-up column (10 × 4.0 mm) filled with Silica CN (20 μm particle size). Following rinsing the clean-up column for 5 min with water containing 8% acetonitrile, analytes were transferred onto the analytical column. Calibration control samples were prepared in a concentration range of 10–600 ng mL^{-1} serum. For quantitative analysis of amisulpride in brain tissue, 750–900 mg of brain tissue was homogenized in 3-fold volumes of methanol using an ultra-turrax (IKA Labortechnik, Staufen, Germany). The homogenates were centrifuged at 13 000 *g*, the supernatants were evaporated to dryness and reconstituted with borate buffer, pH 9.0. The reconstituted samples were subjected to HPLC analysis with fluorescence detection according to a published method (Sachse et al 2003). Calibration standards were prepared by homogenisation of 250 mg brain from untreated rats with 750 mL methanol containing amisulpride resulting in final concentrations of 1–100 ng amisulpride per mL homogenate. The detection limit for UV detection was 10 ng mL^{-1} with an inter-assay precision of 11.3% for 20 ng mL^{-1}, 8.6% for 60 ng mL^{-1}, 4.6% for 150 ng mL^{-1}, and 2.9% for 400 ng mL^{-1}. For fluorescence detection the detection limit was 1.0 ng g^{-1} with an intra-assay precision of 12.5% for 10 ng g^{-1} and 9.1% for 50 ng g^{-1}, respectively.

Fluphenazine was analysed using RP-HPLC and electrochemical detection modified from a method by Cooper et al (1989). Fluphenazine was extracted from serum in the dark using liquid/liquid extraction with ethyl acetate–n-hexane (75:25, v/v). Chlorpromazine served as internal standard. Fluphenazine was extracted from brain tissue using n-hexane–i-amylalcohol (98.5:1.5, v/v). The limit of determination was set to 0.3 ng mL^{-1}. Intra-assay precision was 15.3% at 0.5 ng mL^{-1}, 5.3% at 1.0 ng mL^{-1} and 3.5% at 3.0 ng mL^{-1}. Inter-assay precision was 5.7% at 1.0 ng mL^{-1}. For the assay from brain tissue, the limit of determination was slightly higher (0.35 ng g^{-1}), the intra-assay precision was 12.4% at 1 ng g^{-1}, 4.1% at 10 ng g^{-1} and 9.0% at 100 ng g^{-1}, respectively. The inter-assay precision was 8.9% at 50 ng g^{-1}. There was no chromatographic interference with ciclosporin or its metabolites.

The processing of the calibration standards was identical to the unknown samples. Peak heights obtained from the analysis of calibration samples in either serum or brain tissue homogenates were subject to linear regression analysis. At least three rats were used at each time point and treatment, respectively.

Statistical treatment of data

Significant differences between group means were calculated using the *t*-test for unpaired data when variances were equal between groups. In the case of unequal variances, the Mann–Whitney *U*-test was employed. Calculations were carried out using S-Plus 2000.

Results

All of the studied psychoactive compounds showed affinity to P-gp as demonstrated by the inhibition of [^{3}H]talinolol-mediated transport across Caco-2 monolayers. The IC50 values of transport inhibition are given in Table 1. They ranged from 0.8 μM (quetiapine) to 902 μM (olanzapine). A linear relationship was found between the magnitude of inhibition of [^{3}H]talinolol transport and the IC50 values from the radioligand binding assay (Figure 1). The results of a typical displacement experiment are shown in Figure 2. The lipophilicity

Table 1 IC50 values of psychoactive compounds and verapamil for the inhibition of P-gp-mediated talinolol efflux across Caco-2 monolayers

Compound	Concentration range (μM)	IC50 Talinolol transport (μM)
Domperidone	0–150	45.64 ± 0.17
Amisulpride	0–250	123.50 ± 1.40
Clozapine	0–250	92.78 ± 1.13
Desmethyl clozapine	0–250	125.90 ± 0.50
Fluphenazine	0–100	33.88 ± 1.50
Flupentixol	0–100	18.32 ± 0.04
Fluvoxamine	0–250	146.70 ± 1.95
Perazine	0–250	43.26 ± 0.76
Desmethyl perazine	0–250	166.10 ± 0.15
Perphenazine	0–100	15.45 ± 0.18
Haloperidol	0–20	12.43 ± 0.03
Levomepromazine	0–250	23.04 ± 0.19
Olanzapine	0–250	>250 (902.40 ± 2.60)[a]
Quetiapine	0–250	0.80 ± 0.05
Verapamil	0–50	15.37 ± 0.07

[a]Due to solubility limitations, an accurate experimental determination of IC50 was not possible. Values are the means ± s.d., n = 3.

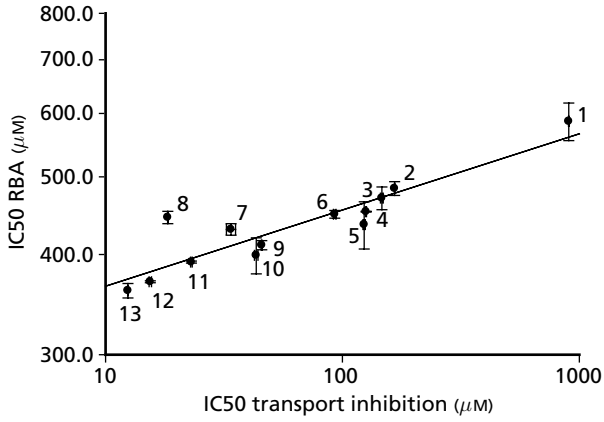

Figure 1 Correlation of P-gp binding as obtained from radioligand-binding assay (IC50 RBA) and transport inhibition studies (IC50 transport inhibition). The dots represent the following compounds: 1, olanzapine; 2, desmethyl perazine; 3, fluvoxamine; 4, desmethyl clozapine; 5, amisulpride; 6, clozapine; 7, fluphenazine; 8, flupentixol; 9, domperidone; 10, perazine; 11, levomepromazine; 12, perphenazine; 13, haloperidol. The error bars represent the experimental variabilities of both variables. The linear regression equation of the log transformed x- and y-variables is: log y = 2.467 + 0.0951*log x ($r^2 = 0.8237$; $P < 0.0001$).

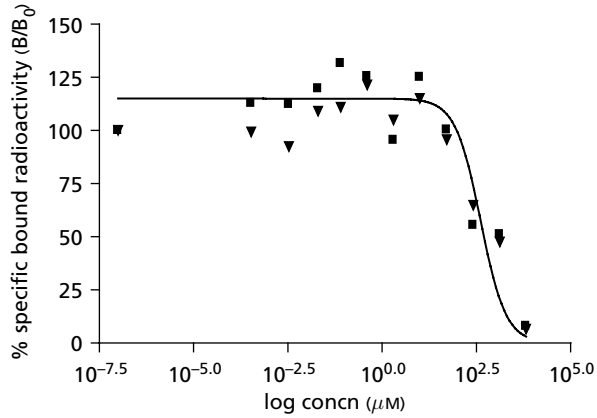

Figure 2 Example of a displacement curve of the radioligand [^{3}H]talinolol by the competitor desmethyl clozapine. Twelve competitor concentrations in the range 0–6553 μM of competitor were studied. Individual data including a common fit using a sigmoidal concentration–response model with variable slope are presented. For each concentration of competitor n = 2.

Table 2 log P and Δlog P values of psychoactive test compounds by potentiometric titration using n-octanol–water and cyclohexane– or dodecane–water systems at 25°C

Compound	log P		Δlog P
	n-Octanol	Cyclohexane/ dodecane	
Fluphenazine	4.50 ± 0.03	2.39 ± 0.04	2.11
Flupentixol	4.27 ± 0.05	2.50 ± 0.02	1.77
Perphenazine	4.29 ± 0.06	0.89 ± 0.09	3.40
Perazine	3.74 ± 0.02	2.91 ± 0.04	0.83
Desmethyl perazine	4.13 ± 0.03	1.32 ± 0.09	2.81
Levomepromazine	4.45 ± 0.01	3.33 ± 0.03	1.17
Clozapine	3.44 ± 0.02	1.40 ± 0.04	2.04
Desmethyl clozapine	3.07 ± 0.04	0.86 ± 0.17	2.21
Fluvoxamine	3.42 ± 0.04	1.39 ± 0.15	2.03
Domperidone	3.89 ± 0.01	1.33 ± 0.05	2.56
Olanzapine	2.89 ± 0.01	0.88 ± 0.13	2.01
Haloperidol	3.49 ± 0.04	n.d.	n.d.
Amisulpride	1.70 ± 0.02	0.23 ± 0.01	1.47
Quetiapine	2.59[a]	n.d.	n.d.

n.d., not determined; [a]determined according to modified HPLC procedure, n = 1 (Abou El Ela 2003). The ionic strength of the aqueous phase was adjusted by the addition of KCl to yield a final concentration of 0.15. The dodecane–water system was used for perazine dimalonate, clozapine, desmethyl clozapine and domperidone since it prevented precipitation of substances during the determination of log P as a result of the volatility of cyclohexane. Given are means ± s.d. of the parameters (n = 3–5).

parameters of the investigated compounds are given in Table 2; log P values ranged between 1.56 (amisulpride) and 4.50 (fluphenazine) in the n-octanol–water system. There was generally a good agreement between measured and predicted log P values by the QMPR software. In no instance did the deviation between the measured and predicted log P values exceed 0.5 log units.

Neither log P nor Δlog P predicted the P-gp inhibitory potency of the psychoactive compounds. With regard to the talinolol transport inhibition assay, R^2 from linear

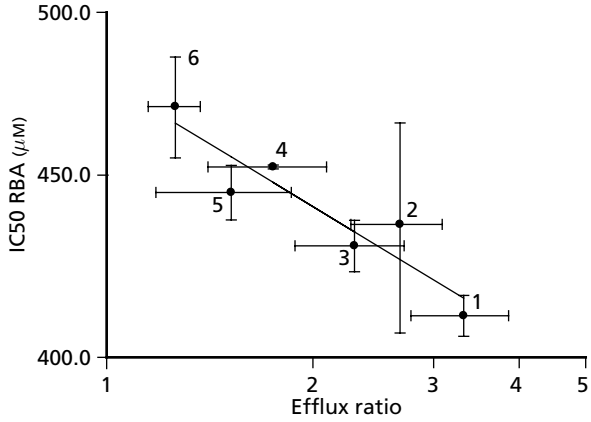

Figure 3 Correlation between IC50 by RBA and transporter-mediated secretion (efflux ratio) of psychoactive compounds in Caco-2 cells. Axes scaling is logarithmic. The numbers refer to the following compounds: 1, domperidone; 2, amisulpride; 3, fluphenazine; 4, desmethyl clozapine; 5, flupentixol; 6, fluvoxamine. Efflux ratio is defined as $(P_{eff}\ b-a)/(P_{eff}\ a-b)$. Given are means ± s.d. of IC50 values and efflux ratios. The variance of the efflux ratios was calculated using the Gaussian error propagation law. The equation for the logarithmically transformed variables is $\log y = -0.1169*x + 2.6796$, $R^2 = 0.8622$. The slope is significantly different from zero ($P < 0.0001$).

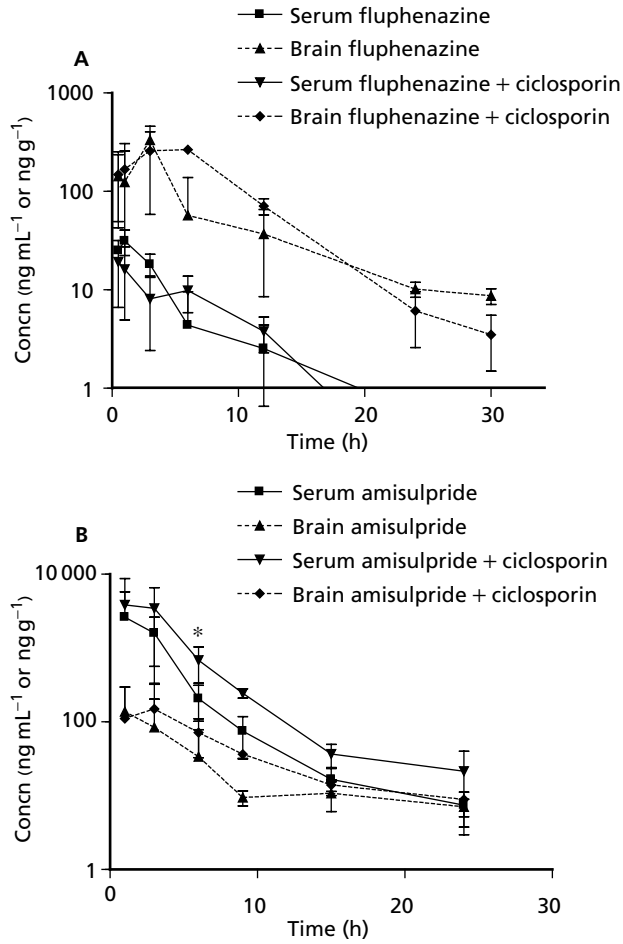

Figure 4 Serum (ng mL^{-1}) and brain (ng g^{-1}) concentration-versus-time profiles of fluphenazine (A) and amisulpride (B) with and without co-administration of ciclosporin. Each time point represents the mean of 3–5 rats ± s.d. For fluphenazine, $P = 0.1$ at the 6- and 30-h time points in brain. For amisulpride, P-values were 0.05, 0.1 and 0.1 at t = 6 h, 9 h and 15 h, respectively, in serum, and $P = 0.1$ at t = 9 h in brain.

regression analysis of log IC50 versus Δlog P amounted to 0.0 whereas the correlation of log IC50 versus log P resulted in an R^2 of 0.3196. Similar results were obtained for the correlation of lipophilicity parameters and IC50 values from the radioligand displacement assay. R^2 amounted to 0.1159 for the correlation of log IC50 versus log P whereas it was 0.0 for the correlation of log IC50 versus Δlog P.

Six of the 14 compounds were identified as P-gp substrates due to the fact that their efflux ratios were significantly greater than 1. The compounds with their corresponding efflux ratios were domperidone (3.32), amisulpride (2.68), fluphenazine (2.30), desmethyl clozapine (1.75), flupentixol (1.52) and fluvoxamine (1.26). A good correlation was found between the P-gp affinity and the extent of P-gp-mediated efflux (efflux ratio), indicating that a high intensity of substrate binding to P-gp triggers efficient carrier-mediated efflux (Figure 3).

For in-vivo studies, the substrates fluphenazine and amisulpride were selected and brain as well as serum concentrations over time were followed after intraperitoneal dosing of 1 mg kg^{-1} of fluphenazine and 50 mg kg^{-1} amisulpride, both alone and in combination with ciclosporin (50 mg kg^{-1}). The respective concentration-versus-time profiles are shown in Figure 4. In the case of amisulpride, the serum concentrations were higher when amisulpride was co-administered with ciclosporin, reaching statistical significance at 6, 9 and 15 h post application, respectively. The difference between the treatments was not as expressed in terms of brain concentrations. Only at 9 h following administration, the brain concentrations of amisulpride were significantly higher for the drug combination as compared with the single administration of amisulpride. Interestingly, the brain-to-serum ratios for amisulpride were not significantly different between the treatment groups (Table 3B), indicating that the higher brain concentrations for amisulpride were in part due to increased systemic serum levels of the drug. In the case of fluphenazine, no significant differences in serum concentrations were observed between administration of fluphenazine alone versus the combination with ciclosporin. However, concentrations of fluphenazine in brain compared with serum concentrations were higher up to 24 h after drug administration for the drug combination regimen. Accordingly, the area under the concentration time curve (AUC) of fluphenazine brain tissue concentrations was 1.6-fold higher, indicating a higher degree of brain accumulation of the antipsychotic drug. This phenomenon is also demonstrated by comparing the brain-to-serum ratios of fluphenazine in the absence and presence of ciclosporin (Table 3A). Except

Table 3A Brain-to-serum ratios for fluphenazine with and without co-administration of ciclosporin to rats

Ratio b/s	0.5 h	1 h	3 h	6 h	12 h	24 h	30 h
Fluphenazine	5.7	3.9	18.3	12.2	14.8	16.8	72.5
Fluphenazine + ciclosporin	8.0	10.2	31.9	27.0*	18.7	30.5	35.0

b/s, concentration of fluphenazine in brain/concentration in serum. *$P < 0.05$ vs fluphenazine alone.

Table 3B Brain-to-serum ratios for amisulpride with and without co-administration of ciclosporin

Ratio b/s	1 h	3 h	6 h	9 h	15 h
Amisulpride	0.048	0.06	0.224	0.867	0.786
Amisulpride + ciclosporin	0.046	0.047	0.133	1.02	1.03

b/s, concentration of amisulpride in brain/concentration in serum.

for the 30-h time point, brain-to-serum ratios were always higher for the combination treatment, reaching statistical significance at 6 h post dosing.

Discussion

Increasing evidence suggests that antipsychotic drugs are able to inhibit carrier-mediated transport processes or are substrates of carriers, in particular efflux transporters from the ABC superfamily (Kataoka et al 2001; Härtter et al 2003; Uhr et al 2003). Little is known about the specific recognition of antipsychotics by P-gp and, furthermore, about the implications of the in-vitro findings on pharmacokinetics, in particular drug–drug interactions (Boulton et al 2002).

In this study a set of experimental methods has been employed to characterize the P-gp affinity of 14 selected drugs that have been used in antipsychotic therapy for several years, (such as haloperidol) or that were introduced to this therapeutic field more recently (quetiapine). The combination of methods employed in the present investigation was designed to discriminate between P-gp inhibitors and substrates within that given set of compounds. Furthermore, correlations between physicochemical parameters of these drugs and their P-gp affinity were supposed. Finally, the relevance of the in-vitro findings with respect to in-vivo pharmacokinetics should be clarified for selected P-gp substrates.

All of the studied antipsychotics show different degrees of affinity towards P-glycoprotein, which manifests itself, for example, by the differences in their inhibitory potency. This observation is not surprising, since various authors (e.g. Stouch & Gudmundsson (2002)) have concluded that, despite the structural heterogeneity of modulators of multi-drug resistance, these substances share the common property of high lipophilicity and further contain a nitrogen atom that is positively charged at physiological pH. All of the compounds investigated in this study fulfill that requirement, although they belong to chemically different classes: tricyclic phenothiazines and phenothiazine-analogues (thioxanthines), such as fluphenazine, flupentixol, perphenazine, perazine, desmethyl perazine and levomepromazine, dibenzodiazepines (clozapine and its metabolite desmethylclozapine), oximes (fluvoxamine), benzimidazoles (domperidone), dibenzodiazepines (olanzapine), butyrophenones (haloperidol), benzamides (amisulpride) and dibenzothiazepines (quetiapine). Common to all of these compounds is their relatively high lipophilicity with log P values ranging from 1.7 (amisulpride) to 4.5 (fluphenazine). All of them contain nitrogen atoms with at least one pKa value in the neutral to alkaline pH range, thus demonstrating their, at least partial, positive charge at physiological pH values. For several of the piperazine-containing compounds, an NH-acidic group is also present. The compounds do not differ to a considerable extent in terms of molecular weight, ranging from 311.8 (desmethylclozapine) to 437.5 (fluphenazine). The clinical significance of the in-vitro P-gp-inhibition data needs to be established in the future. It would seem that antipsychotics with high P-gp inhibition potency have the most prominent tendency to modify the pharmacokinetics of other P-gp substrates. In that respect, quetiapine is an interesting compound since its IC50 for inhibiting talinolol efflux is lowest among all antipsychotics studied. On the other hand, quetiapine is also metabolized by CYP 3A4 (Prior & Baker 2003), such that the co-administration of other substrates or inhibitors of CYP 3A4 (e.g., HIV protease inhibitors, azole antimycotics or erythromycin) together with quetiapine is contra-indicated. Since several CYP 3A4 inhibitors and substrates are also P-gp inhibitors and substrates, it is impossible to discriminate between the different mechanisms — metabolism or transport — for the interaction. Future studies on the interaction with highly potent P-gp inhibitors among antipsychotics and metabolically fairly stable P-gp substrates (e.g. digoxin, fexofenadine or talinolol) would help to answer that particular question.

In addition to P-gp binding and displacement experiments, P-gp substrates have been identified based on their efflux ratios and on its susceptibility with respect to the presence of verapamil. Two of the six substrates identified here have been reported as P-gp substrates in earlier publications and with alternative experimental techniques. Schinkel et al (1996) reported that domperidone is an excellent P-gp substrate using in-vivo investigations in mice. Moreover, specific patterns for the substrate recognition by P-gp have been postulated (Seelig 1998; Seelig & Landwojtowicz 2000). These patterns consist of hydrogen bond acceptor (or electron donor) groups (e.g. carbonyl, ether, hydroxy or nitrogen contained in imines [-N=], in tertiary [-NR_2], secondary [-NHR], or primary [-NH_2] amines, phenyl groups and certain halides) with a specific spatial separation. Recognition patterns formed by two electron donor groups with a spatial separation of 2.5 ± 0.3 Å are called type-1 patterns. Recognition elements

formed by two electron donor groups with a spatial separation of 4.6 ± 0.6 Å are called type-2 patterns. For binding to P-gp, at least one type-1 or one type-2 pattern is required and binding is shown to increase with the strength and number of electron donor groups involved in type 1 and type 2. For transport via P-gp, at least two type-1 patterns or one type-1 and one type-2 pattern are considered to be necessary (Seelig 1998; Seelig & Landwojtowicz 2000). Domperidone contains two type-1 recognition patterns with a special separation of 2.5 ± 0.3 Å and thus confirms the predictive value of the model. Similarly, flupentixol, as well as its structural homologue, fluphenazine, have been identified as P-gp substrates (Ford et al 1989), a property that can also be predicted on the basis of the Seelig model.

Neither binding to P-gp nor the magnitude of efflux ratio show satisfactory correlations with the lipophilicity parameter, log P, or with the number of hydrogen bond donors and acceptors expressed experimentally as Δlog P in this study. This somewhat contradictory finding in relation to other published data (Koggel 2002; Leisen et al 2003) may be due to the fact that, here, 11 out of 14 compounds are very lipophilic (log $P > 3$) and thus many of the compounds investigated in this study are physicochemically not sufficiently different to represent a balanced data set.

On the other hand, this study has demonstrated for the first time a significant positive correlation between the binding affinity of P-gp substrates and the efficiency of their carrier-mediated transport, expressed as efflux ratio. This underlines the predictive value of the radioligand displacement method when screening for P-gp affinity of xenobiotics. However, more research with different sets of compounds will be needed before the method can be generally recommended as a primary predictive screen for P-gp affinity and drug–drug interactions.

Based on the outcome of the in-vitro permeability experiments, 6 of the 14 investigated compounds were identified as P-gp substrates. It may be discussed whether a compound not showing an efflux ratio significantly greater than one in the Caco-2 permeability model may under all circumstances be classified as a non-P-gp substrate. At least for olanzapine, which has an efflux ratio of only 1.1 and a weak affinity to P-gp in the models employed here, evidence has been presented in a P-gp deficient knockout mouse model that its entry into brain and other organs protected by P-gp is increased in the Abcb1a $(-/-)$ mice as compared to the wild-type controls (Wang et al 2004). The underlying reason for this discrepancy is currently not known. For the antipsychotics classified as P-gp substrates, it was anticipated that the co-administration of a P-gp inhibitor such as ciclosporin together with a P-gp substrate would modify the pharmacokinetics of the substrate. Based upon the localization of P-gp in the endothelium of the brain capillary cells and in the liver and kidneys, changes both in the CNS distribution as well as in the excretion of the substrate have been predicted. Whereas the former process would lead to increased brain concentrations of the P-gp substrate, it is expected that the latter will generally lead to increased serum concentrations of the substrate due to the partial blockade of an excretion pathway. With respect to fluphenazine, this hypothesis has been confirmed by the outcome of the in-vivo experiments. Significantly increased brain-to-serum concentration ratios were measured over the whole concentration–time profile (except for the last time point, 30 h after drug administration). This explanation follows the line of thought outlined by Döppenschmitt et al (1998). Interestingly, there were no significant differences in the serum concentrations of fluphenazine when it was administered alone and in combination with ciclosporin. Fluphenazine is a CYP2D6 inhibitor and more than 90% of the compound administered parenterally is metabolised. Therefore, a drug–drug interaction due to the blockade of an excretion pathway for the parent compound is unlikely to occur, due to the fact that the major part of the compound is eliminated via metabolism. Amisulpride, on the other hand — in the series of compounds investigated — represents the most hydrophilic drug with the lowest membrane permeability. It is primarily excreted unchanged into the urine and undergoes fairly little metabolism. In this case, co-administration of a P-gp inhibitor will clearly have an effect on the clearance of amisulpride and thus leads to significantly increased serum and brain concentrations of the drug when co-administered with ciclosporin.

It may be concluded that drug–drug interactions based on the in-vitro competition between transporter substrates and inhibitors may or may not have in-vivo relevance. Thus the value of in-vitro techniques in the prediction of an in-vivo drug–drug interaction will depend on all pharmacokinetic processes of the drug and may not be broken down to a single event such as the interaction of the compound with one specific transporter or enzyme. Only in such cases where a drug transporter interaction is dominant for the overall pharmacokinetics of the drug, will an inhibition of that process lead to significant changes in the overall pharmacokinetics and potentially also the effect.

Conclusions

Drug–drug interactions between P-gp substrates and inhibitors during the disposition phase may affect the tissue distribution and excretion of therapeutically important antipsychotic drugs. In-vitro measurements of membrane permeability in P-gp-containing Caco-2 cells and affinity studies with transporter cell preparations may be a useful predictor of such interactions.

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