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Inhibition of P-glycoprotein by newer antidepressants

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Running title: Antidepressants and Pgp

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Abbreviations:

BCECs, brain capillary endothelial cells; pBCECs, porcine BCECs; calcein-AM, calcein-acetoxymethylester; CNS, central nervous system; CYPs, cytochrome P450 enzymes; DMSO,

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dimethyl sulfoxide; FCS, foetal calf serum; f2, concentration needed to double baseline fluorescence; GFAP, glial fibrillary acidic protein; HBSS, Hank's balanced salt solution; HIV, human immunodeficiency virus; HHBSS, HBSS supplemented with 10 mM HEPES; IC_{50}, concentration leading to half maximal inhibition; IP_{50}, inhibitory potency at 50 µM; MDR, multidrug resistance; MRP, multidrug resistance-associated protein, paroxetine-M, paroxetine metabolite; Pgp, P-glycoprotein; ppgp, porcine pgp; SSRIs, selective serotonin reuptake inhibitors; RT-PCR, reverse transcription-polymerase chain reaction; v, volume; VPL, verapamil.

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Abstract

Pharmacokinetic drug-drug interactions often occur at the level of P-glycoprotein (Pgp). To study possible interactions caused by the newer antidepressants we investigated citalopram, fluoxetine, fluvoxamine, paroxetine, reboxetine, sertraline, and venlafaxine and their major metabolites desmethylcitalopram, norfluoxetine, paroxetine-metabolite (paroxetine-M), desmethylsertraline, N-desmethylvenlafaxine, and O-desmethylvenlafaxine for their ability to inhibit Pgp. Pgp inhibition was studied by a fluorometric assay using calcein acetoxymethylester (calcein-AM) as Pgp substrate and two different cell systems: L-MDR1 cells (model for human Pgp) and primary porcine brain capillary endothelial cells (pBCECs, model for the blood-brain barrier). Both cell systems proved to be suitable for the evaluation of Pgp inhibitory potency of drugs. All antidepressants tested except O-desmethylvenlafaxine showed Pgp inhibitory activity with sertraline, desmethylsertraline, and paroxetine being the most potent, comparable to the well known Pgp inhibitor quinidine. In L-MDR1 cells fluoxetine, norfluoxetine, fluvoxamine, reboxetine, and paroxetine-M revealed intermediate Pgp inhibition and citalopram, desmethylcitalopram, venlafaxine, and N-desmethylvenlafaxine were only weak inhibitors. The ranking order was similar in pBCECs. The fact that some of the compounds tested exert Pgp inhibitor effects at similar concentrations like quinidine suggests that pharmacokinetic drug-drug interactions between the newer antidepressants and Pgp substrates should now be thoroughly studied *in vivo*.

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P-glycoprotein (Pgp) is a member of the ATP-binding cassette (ABC) superfamily of membrane transport proteins, responsible for the efflux of many drugs. It represents a major component of the blood-brain barrier (Schinkel et al., 1994), the intestinal barrier (van Asperen et al., 1998) and contributes to renal and biliary elimination of drugs (Kusuhara et al., 1998; Chiou et al., 2000). At the blood-brain barrier Pgp is localized in the apical membrane of brain capillary endothelial cells (BCECs) and transports substrates towards the blood compartment (Cordon-Cardo et al., 1989; van Asperen et al., 1997). Therefore, Pgp can limit the penetration into and retention within the brain and thus modulate effectiveness and central nervous system (CNS) toxicity of numerous compounds. In contrast, the absence of active Pgp as observed in mdr-1 knockout mice lacking Pgp and thus exhibiting unrestricted access of Pgp substrates to the brain yields significantly increased CNS concentrations often exceeding those observed in wild-type mice by orders of magnitude (Schinkel et al., 1994; Schinkel et al., 1996). Pgp is also highly expressed in the apical membrane of epithelial cells in the small and large intestine, where it transports drugs out of the cells into the intestinal lumen (Cordon-Cardo et al., 1989; van Asperen et al., 1998) thus limiting bioavailability of compounds like paclitaxel and HIV protease inhibitors (Sparreboom et al., 1997; Kim et al., 1998).

Established antidepressants, in particular tricyclic antidepressants have a significant potential of inducing adverse drug reactions, exhibit a limited effectiveness even in patients with optimum compliance (Bollini et al., 1999), and are subject to numerous drug-drug interactions (Stockley, 1999). Newer antidepressants, which have been developed and introduced to supplement the established antidepressants, include the selective inhibitors of serotonin reuptake (SSRIs, e.g. sertraline) or norepinephrine reuptake (e.g. reboxetine) or compounds inhibiting reuptake of both neurotransmitters (e.g. venlafaxine). They are almost completely biotransformed before excretion (Caccia, 1998) and some of the numerous known metabolites

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(e.g. norfluoxetine, N- and O-desmethylvenlafaxine) are active with significant antidepressant effects (Caccia1998; Preskorn, 1997).

Because it is impossible to perform drug-drug interaction studies with all combinations prescribed to patients, it is important to elucidate possible mechanisms of interaction *in vitro* in order to guide initial dosage adaptations and / or to prompt focussed interaction studies *in vivo* and efficient monitoring of adverse drug reactions in patients.

The role of Pgp in causing clinically relevant drug interactions is becoming more and more obvious (Yu, 1999). Concentrations of paroxetine and venlafaxine increased significantly (1.7 to 3 fold) in the brain of *mdr1ab* (-/-) knockout mice after single dose administration and after treatment for 11 days (Uhr, 2002) suggesting, that these antidepressants are Pgp substrates and that their pharmacokinetics might be influenced by co-administered Pgp inhibitors. In contrast, for fluoxetine the absence of Pgp substrate characteristics was reported (Uhr et al., 2000), indicating that not all class members share these properties. For citalopram, the results are contradictory (Rochat et al., 1999; Uhr et al., 2000). Hitherto, nothing is known about the inhibitory potential of the newer antidepressants on Pgp and thus their potential to modulate the access of Pgp substrates to the brain.

We therefore characterized the inhibitory potencies of the newer antidepressants and some of their available main metabolites in an *in vitro* assay by using calcein-acetoxymethylester (calcein-AM) as Pgp substrate. Two different cell systems expressing Pgp were used and compared concerning their suitability for a Pgp inhibition assay: L-MDR1 (porcine kidney cells overexpressing the human isoform of the transporter Pgp) (Schinkel et al., 1995) and primary cell cultures of porcine brain capillary endothelial cells (pBCECs), expressing porcine pgp1A (Török et al., 1999) as a model for the blood-brain barrier.

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Methods

Materials

Culture media, foetal calf serum, medium supplements, antibiotics and HBSS were purchased from Invitrogen (Karlsruhe, Germany), Collagenase/Dispase and Dispase were from Roche Diagnostics (Mannheim, Germany), Collagen-R from Serva (Heidelberg, Germany), DMSO and Triton X-100 were from AppliChem (Darmstadt, Germany), Dextran from Sigma-Aldrich (Taufkirchen, Germany), Percoll® from Amersham Biosciences (Freiburg, Germany), Calcein-AM from MoBiTec (Göttingen, Germany), vincristine from Calbiochem (Darmstadt, Germany), 96-well microtiter plates were from Nunc (Wiesbaden, Germany).

Drugs

Citalopram hydrobromide and desmethylcitalopram hydrochloride were kind gifts from Lundbeck (Valby, Denmark), fluvoxamine maleate from Solvay (Hannover, Germany), LY335979 was obtained from Eli Lilly Company (Bad Homburg, Germany), paroxetine hydrochloride hemihydrate and paroxetine metabolite from GlaxoSmithKline (Stevenage, United Kingdom), SDZ-PSC833 from Novartis (Basel, Switzerland), reboxetine methanesulphonate from Pharmacia (Kalamozoo, USA), sertraline hydrochloride and desmethylsertraline from Pfizer (Karlsruhe, Germany), venlafaxine hydrochloride, N-desmethylvenlafaxine hydrochloride, and O-desmethylvenlafaxine from Wyeth (Münster, Germany). Fluoxetine hydrochloride, norfluoxetine hydrochloride, and verapamil hydrochloride were purchased from Sigma-Aldrich (Taufkirchen, Germany) and quinidine from Roth (Karlsruhe, Germany).

LLC-PK1 and L-MDR1 cells

As model for human Pgp we used L-MDR1 cells, a cell line generated by transfection of the porcine kidney epithelial cell line LLC-PK1 with the human *MDR1* gene (Schinkel et al.,

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1996) and the parental cell line LLC-PK1 (available at ATCC, Manassas, U.S.A.) as a control. The L-MDR1 cell line was kindly provided by Dr. A. H. Schinkel (Amsterdam, the Netherlands). The cells were cultured under standard cell culture conditions with M199 supplemented with 10% heat inactivated foetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate. To maintain Pgp expression, the culture medium for L-MDR1 was supplemented with 0.64 µM vincristine. For the calcein assay, cells were seeded on collagen-coated microtiter plates in a density of 10.000 cells/cm^2 and cultured for three days. One day before the assay, both cell lines were fed with vincristine-free culture medium.

Isolation of porcine brain capillary endothelial cells

The isolation of pBCECs was essentially based on the method described by Audus and co-workers (Audus et al., 1996). M199 supplemented with L-glutamine (0.7 mM), streptomycin sulfate (100 µg/ml), penicillin G (100 U/ml), gentamycin (100 µg/ml), and HEPES (10 mM) was used for all preparation steps. Briefly, cortical gray matter from seven to eight fresh porcine brains, which were obtained from a local slaughterhouse, was isolated, cut into very small pieces and digested enzymatically using 0.5% dispase (in preparation medium). After centrifugation (1000 x g, 4 °C, 10 min), the pellets were resuspended in 13% dextran solution in preparation medium and centrifuged again (5800 x g, 4 °C, 12 min). The pellet containing the cerebral microvessels was subsequently incubated in preparation medium containing 0.1% (w/v) collagenase/dispase. The resulting cell suspension was filtered and the BCECs were separated on a discontinuous Percoll® gradient (densities 1.03 and 1.07; centrifugation at 1250 x g, 5 min, 20 °C), washed and filtered again before being seeded on collagen-coated microtiter plates in a density of 100.000 cells/cm^2. Cells were cultured under standard cell culture conditions with M199 containing L-glutamine (0.7 mM), streptomycin sulfate (100

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µg/ml), penicillin G (100 U/ml), HEPES (10 mM), and 10% heat-inactivated horse serum. Eight days after seeding the confluent monolayers were used for the calcein assay.

The isolation method for the pBCECs was validated by immunohistochemistry. The isolated cells were positive for the endothelium-specific factor VIII–related protein (von Willebrandt factor) and for Pgp. Potential contamination with astrocytes was assessed by staining with an antibody against glial fibrillary acidic protein (GFAP) and was negligible (<5%). Staining with an antibody against neurofilaments (as a marker for neurons) was absent.

RT-PCR for the detection of Pgp expression at the mRNA level

Expression of human Pgp and/or porcine pgp1A (ppgp1A) at the mRNA level in the cell lines used was verified by reverse transcription of RNA followed by polymerase chain reaction (RT-PCR). RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). First strand cDNA synthesis was performed with the 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Mannheim, Germany) with random hexamer primers according to the manufacturers instructions.

Primers used for the amplification of human Pgp were 5’-GTGCTGGTTGCTGCTTACAT-3’ (sense) and 5’-CCCAGTGAAAAATGTTGCCA-3’ (antisense). For ppgp1A, primers were employed according to Childs & Ling (Childs and Ling, 1996). All primers were synthesized by MWG Biotech AG (Ebersberg, Germany). PCR was performed on the LightCycler™ (Roche Diagnostics, Mannheim, Germany) in a total volume of 10 µl using the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany), 0.5 mM of each primer and 3 mM $MgCl_2$. PCR fragment size was determined by 1.5% agarose gel electrophoresis.

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Stock solutions

Stock solutions of test compounds were prepared strictly following the manufacturers instructions. Most compounds were soluble in aqua bidest. Only sertraline, desmethylsertraline, O-desmethylvenlafaxine, quinidine, verapamil hydrochloride, SDZ-PSC833, and LY335979 were dissolved in DMSO. The DMSO concentration in the assays never exceeded 1% (v/v), a concentration which was found not to influence the results of the assay in pilot experiments.

Calcein uptake assay

Calcein-AM is a fluorogenic, highly lipid-soluble dye which rapidly penetrates the plasma membrane. Inside the cell, endogenous esterases cleave the ester bonds, producing the hydrophilic and fluorescent dye calcein, which cannot leave the cell via the plasma membrane (Hollo et al., 1996). Whereas calcein-AM is a substrate of Pgp, calcein is not (Hollo et al., 1996; Homolya et al., 1993). Cells expressing high levels of Pgp rapidly extrude nonfluorescent calcein-AM from the plasma membrane, thus preventing accumulation of fluorescent calcein in the cytosol. Because the transport capacity of Pgp is inversely proportional to the accumulation of intracellular calcein fluorescence, inhibition of Pgp will lead to intracellular calcein accumulation.

The calcein-AM uptake assay was performed in 96 well plates. All incubation steps and the cell lysis were conducted at 37 °C on a rotary shaker at 450 rpm. Prior to the uptake assay, the cells were washed with prewarmed HBSS supplemented with 10 mM HEPES (HHBSS) and preincubated with HHBSS for 30 min and subsequently with the test compound for 10 min in octuplet. After preincubation, calcein-AM was added (final concentration 1 µM) and the cells were incubated for 60 min. The uptake was then stopped by transferring the plates on ice and washing the cells twice with HHBSS precooled to 4 °C. Subsequently, cells were lysed in 1% Triton X-100 for 15 min. The fluorescence of the calcein generated within the cells was

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analyzed in a Fluoroskan Ascent fluorometer (Labsystems, Frankfurt, Germany) with 485 nm excitation and 535 nm emission filters. Each experiment was performed at least in triplicate on different days.

Quenching test

Each test compound was analyzed for possible quenching effects on the calcein fluorescence. Since calcein-AM is nonfluorescent and cannot be used for a quenching test, we generated the fluorescent dye calcein by incubating LLC-PK1 cells with 1 µM calcein-AM for 60 min at 37 °C on a rotary shaker at 450 rpm. Increasing concentrations of the test compounds were added to aliquots of the cell lysate and the fluorescence was compared to control wells without test compounds.

Cytotoxicity assay

Each test compound was screened for possible cytotoxic effects with the Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany), a colorimetric assay for the quantification of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant.

Statistical analysis

For calculation of the inhibitor effects, a non-linear four parameter fit was used (Grafit, version 4, Erithacus Software, Middlesex, UK) according to the sigmoidal I_{max} model with the following formula:

$y = ((I_{max} - \text{Background})/(1+(x/IC_{50})^{s})) + \text{Background}$,

where I_{max} is the maximal inhibition, IC_{50} is the concentration leading to half-maximal inhibition of the calcein-AM transport, and s is the slope factor.

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Due to solubility problems and/or cytotoxic influences, plateau effects and thus IC_{50} values were only obtained for some of the compounds tested. The curves were therefore also evaluated with two additional methods which are not based on plateau effects. In the first analysis the concentration of the test compound needed to double baseline fluorescence was derived from the concentration-response curve (f2) (Figure 1). The second calculation quantifies the inhibitory effect of the compound on calcein-AM efflux at a fixed concentration (50 µM for all compounds except SDZ-PSC833 [2.5 µM] and LY335979 [1 µM]) and normalizes this value to the maximal effect of verapamil (VPL) control in the same assay. The resulting IP_{50rel} was calculated with the following formula (Bogman et al., 2001)

$$IP_{50rel} = \Delta F_{\text{test compound at 50 µM}} / \Delta F_{\text{VPL at 200 µM}},$$

where ΔF is the difference in calcein fluorescence in the absence and presence of the Pgp inhibitor.

P-values were determined by ANOVA with Dunnett’s multiple comparison test for post hoc pairwise comparison with the control results obtained with verapamil or with the Wilcoxon matched-pairs test (GraphPad InStat, version 3.05, GraphPad Software, San Diego, USA). A p-value of ≤ 0.05 was considered significant.

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Results

Method validation

RT-PCR demonstrated the expression of mRNA of human Pgp and/or porcine pgp1A (Figure 2). These results were verified by Western blot and immunohistochemistry (data not shown). The different Pgp levels of L-MDR1 and LLC-PK1 were also confirmed in functional experiments by differences in calcein accumulation and its inhibition by verapamil, a typical Pgp inhibitor (Figure 3).

To exclude a possible involvement of the multidrug resistance-associated proteins (MRPs) 1 and 2, that also transport calcein-AM, we tested probenecid up to 500 µM in the calcein assay. Probenecid has been shown to inhibit the transport of calcein-AM and calcein in MRP- but not in Pgp-overexpressing cell lines (Feller et al., 1995; Gollapudi et al., 1997). In L-MDR1 cells probenecid had no influence on the calcein accumulation up to 500 µM (n = 3 experiments in octuplets), in pBCECs only a minor effect could be seen (n = 3 experiments in octuplets, f2 was not reached). Similarly, the specific MRP inhibitor MK571 had no effect in L-MDR1 cell up to 2.5 µM and only a minor effect in pBCECs.

None of the test compounds showed any quenching effect in the concentration range tested on the fluorescence of calcein or an autofluorescence at the excitation wavelength used to measure calcein fluorescence.

In the upper concentration range, most of the antidepressants tested showed cytotoxic effects. The corresponding values leading to a decline in the concentration response curve were not included in the analysis of the calcein assay. The four control compounds SDZ-PSC833, LY335979, verapamil, and quinidine proved not to be cytotoxic for any of the cell lines even in the highest concentrations used.

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The ranking order of the three control compounds SDZ-PSC833, verapamil, and quinidine confirmed the results of the calcein assay conducted by Tiberghien and Loor (Tiberghien and Loor, 1996). The prototype Pgp substrate digoxin showed no inhibition in L-MDR1 cells up to 500 µM and only weak inhibition in pBCECs (f2 = 76.4 ± 15.9 µM, n = 2 experiments in octuplets, concentration of calcein-AM = 0.5 µM).

Evaluation of the Pgp inhibitory potency of the newer antidepressants

All concentration-response curves reaching a plateau and thus enabling the calculation of IC_{50} values are shown in Figure 4. In addition to three of the four control compounds (Figure 4a), this applied to sertraline, paroxetine, and fluoxetine in L-MDR1 cells (Figure 4b, Table 1). The potency of sertraline and paroxetine was comparable to the potency of quinidine (Table 1 and 2). For most compounds plateau effects were not reached either because of cytotoxicity or limited dissolution. To permit a comparison between all compounds tested, IP_{50rel} and f2 values were calculated (Table 1 and 2). Only venlafaxine and its metabolites (in both cell lines) and fluoxetine (in pBCECs) did not reach f2. Nevertheless, except for O-desmethylvenlafaxine the highest concentrations analyzed (100 µM for fluoxetine and O-desmethylvenlafaxine, 500 µM for venlafaxine and N-desmethylvenlafaxine) produced significant increases in baseline fluorescence ($p<0.0001$, Wilcoxon matched-pairs test) thus confirming Pgp inhibition.

Independent from the calculation used and the cell line tested, LY335979, SDZ-PSC833, and verapamil proved to be the most effective Pgp inhibitors followed by quinidine, sertraline, desmethylsertraline, and paroxetine which were all similar (Table 1, Table 2, Figure 5). In L-MDR1 cells fluoxetine, norfluoxetine, fluvoxamine, reboxetine, and paroxetine-M showed intermediate and citalopram, desmethylcitalopram, venlafaxine, and N-desmethylvenlafaxine only weak inhibition. No inhibition was found for O-desmethylvenlafaxine. The results obtained in pBCECs were similar (Table 2).

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Discussion

The activity of the efflux transporter Pgp affects the pharmacokinetic parameters of many drugs and contributes to numerous pharmacokinetic drug-drug interactions (Yu 1999). Hitherto, the role of Pgp for the bioavailability, distribution, and excretion of the newer antidepressants and for their interaction with co-administered drugs has not been elucidated thoroughly. For paroxetine, venlafaxine, and fluoxetine the data indicate, that they might be Pgp substrates, for citalopram the data are conflicting (Rochat et al., 1999; Uhr et al., 2000; Uhr 2002).

Even less is known about potential inhibitory characteristics of newer antidepressants on Pgp. In theory, Pgp inhibition by drugs may play an important role in drug safety, because it may increase plasma and brain concentrations of co-administered drugs and thus cause adverse drug reactions. Thus far, only fluoxetine has been tested in this regard. In line with the absence of Pgp-substrate characteristics (Uhr et al., 2000) and in agreement with our results no evidence for a potent interaction was found (Ekins et al., 2002). So far, there are no studies which systematically examine possible interactions with the newer antidepressants at the level of Pgp.

The aim of the present study was to clarify whether the widely used newer antidepressants and their major metabolites inhibit Pgp *in vitro* as a marker of potential drug-drug interactions *in vivo*. Another objective was the comparison of the pBCECs and L-MDR1 cells concerning their suitability for a Pgp inhibition assay with calcein-AM as Pgp substrate.

We used an *in vitro* assay to characterize compounds concerning their ability to inhibit the transport of the Pgp substrate calcein-AM. The fact, that all four well characterized Pgp inhibitors (LY335979, SDZ-PSC833, verapamil, and quinidine) were also potent Pgp inhibitors in these assays confirms the applicability of the calcein assays for the evaluation of Pgp modulating drug effects. The two cell systems used offer different advantages. L-MDR1

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cells overexpress human Pgp and can be compared to their parental cell line LLC-PK1. Effects only seen in the transfected cell line can thus be attributed to functional human Pgp. Effects observed in both cell lines can be ascribed to porcine pgp1A which is expressed in both cells or to other shared characteristics. Indeed, the substantial difference in baseline fluorescence and the fact that verapamil had nearly no effect in LLC-PK1 cells confirms the expected difference in the Pgp activity between parental and transfected cell line (Figure 2) and emphasizes the suitability of this cell system.

For all drugs tested, the influence on calcein fluorescence in LLC-PK1 cells was either absent or much less pronounced than in the Pgp overexpressing cell line, indicating that the enhancement of the calcein fluorescence was based on inhibition of human Pgp. This finding indicates that MRP1 and 2 do not play a substantial role in this assay, particularly because the MRP inhibitors probenecid and MK571 had no effects in L-MDR1 cells and only minor effects in pBCECs on the calcein accumulation. This conclusion was also drawn by Eneroth and coworkers, evaluating a Pgp overexpressing Caco-2 cell line in a calcein assay (Eneroth et al., 2001).

Interestingly, the maximal fluorescence obtained in L-MDR1 cells was greater than in LLC-PK1 cells (Figure 2), most likely because L-MDR1 cells are roughly 60 % thicker than LLC-PK1 cells and thus accumulation of calcein in the transfected cell line may be greater. Another possibility for such a finding might be differences within the intracellular milieu e.g. involving different esterase activities.

For comparison primary cell cultures of (porcine) pBCECs as a second, independent cell system were studied. These cells are sumptuous to isolate, differ slightly from preparation to preparation and are more sensitive to cytotoxic effects. They exhibit a constant pgp1A expression (Hegmann et al., 1992; Huwyler et al., 1996) and due to the lower Pgp expression level compared to L-MDR1 cells they appear particularly suited to detect minor effects of weak inhibitors.

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The similarity of the ranking order of inhibition in both cell systems suggests, that both are suitable for the evaluation of Pgp modulating effects. However, it is common experience that absolute values cannot be compared between different cell systems (Table 1 and 2), especially if Pgp expression levels are different. Therefore, it is conceivable that different concentrations of a compound are needed in the two cell systems to reach similar effects.

Whenever possible, IC_{50} values were employed to compare inhibitory characteristics. If no plateau effect was reached and solubility or cytotoxic effects precluded further increases of the concentration, we also calculated IP_{50rel} (Bogman et al., 2001) and the concentration needed to increase basal fluorescence twofold (f2). The IP_{50rel} features the advantage of normalizing all values to a control (e.g. verapamil) and thus compensates for inter-assay variability. However, the concentration at which the effect is compared is predefined arbitrarily, neglecting the fact, that the slope factors of different concentration-effect curves may differ and that the potency of different compounds may vary by orders of magnitude.. Moreover, the meaningful comparison of compounds with substantially differing maximum effects (efficacy) is not possible and the IP_{50rel} does not give clear evidence at which concentration range a compound is active.

Hence, we introduced another assessment method (determination of f2) which also takes the different shapes of the respective concentration-response curves into consideration. Only for very weak inhibitors, which do not lead to a twofold increase in basal fluorescence, this method is not suitable. However, such minor effects are normally negligible and may not have importance for the *in vivo* situation. As an example, in the calcein assay the prototype Pgp substrate digoxin has only minor effects. This is perfectly in line with extensive clinical experience with this drug with only insignificant and rare alteration of the pharmacokinetics of co-administered substrates (Rameis, 1985). Despite the individual advantages and disadvantages of the different cell systems applied and independent of the calculation

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methods and the cell line used, this series of experiments for the first time shows, that the newer antidepressant are inhibitors of Pgp. Indeed, sertraline, desmethylsertraline, and paroxetine had an effect similar to one of the most potent inhibitors (quinidine) whereas citalopram, desmethylcitalopram, venlafaxine, and N-desmethylvenlafaxine exerted only very weak inhibition.

Based on these *in vitro* data, sertraline and paroxetine bear the apparently largest potential to influence the pharmacokinetics of co-administered drugs at the level of Pgp. However, at usual therapeutic doses, the IC_{50}-value for inhibition of Pgp is around 250-fold higher than the plasma concentration for paroxetine and around 500-fold higher for sertraline (Preskorn 1997). Von Moltke and co-workers (von Moltke et al., 1998) recently suggested a model to predict *in vivo* drug interactions based on *in vitro* data which they applied to estimate the inhibitor potential of SSRI on cytochrome P450 (CYP) 2D6. Provided that blood:liver concentration ratios were considered, which amount to 1:36 for sertraline, and not the unbound SSRI plasma concentration, the model yielded good predictions of the in vivo situation. For sertraline, with effective plasma levels of about 65 nM (Preskorn, 1996), this would implicate concurrent liver concentrations of about 2 µM. However, these concentrations are roughly one order of magnitude below the concentrations which were found to inhibit Pgp in our assays suggesting that even if the accumulation of the drugs within the cell (e.g. in the biliary or renal system) is taken into account, the Pgp inhibition observed *in vitro* might not be clinically relevant.

This is substantiated by the fact, that neither sertraline (Rapeport et al., 1996) nor fluvoxamine (Ochs et al., 1989), or citalopram (Larsen et al., 2001) had a clinically relevant influence on the pharmacokinetic parameters of digoxin, a Pgp prototype substrate. There is only one case report, describing a possible increase of serum digoxin levels after treatment with fluoxetine (Leibovitz et al., 1998). On the other hand, in addition to being an inhibitor of CYP2D6, paroxetine is a substrate of this isozyme, whose activity is regulated by a genetic

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polymorphism. In the absence of active enzyme (poor metabolizer) plasma paroxetine concentrations are up to 25fold higher than in extensive metabolizers (Sindrup et al., 1992). Accordingly, it can not be excluded, that in poor metabolizer patients administration of high paroxetine doses may translate into clinically relevant modulation of the pharmacokinetics of concomitantly administered Pgp substrates.

In conclusion, the present study demonstrates that not only the widely used L-MDR1 cells are well suited to evaluate drug-induced Pgp inhibition with calcein-AM, but also the pBCEC primary cell cultures which are a well established model to assess the pharmacological properties of the blood-brain barrier. This is the first study which comprehensively quantified inhibitory effects of the newer antidepressants some of which exerted substantial Pgp inhibition. It remains to be investigated whether this property of the newer antidepressants might lead to drug-drug interactions in patients.

Such interactions might for instance be relevant when drugs with low oral bioavailability due to substantial transport back into the gut lumen are to be coadministered, as it has be shown for loperamide when given in combination with quinidine (Sadeque et al., 2000).

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Table 1: Inhibition of Pgp by newer antidepressants and typical Pgp inhibitors in L-MDR1 cells.

Test compound	n =	IC_{50} (µM)	IP_{50rel} (for SDZ-PSC833 $IP_{2.5rel}$ and for LY335979 IP_{1rel})	Concentration (µM) needed to double baseline fluorescence (f2)
SDZ-PSC833	3	n.d.	1.32 ± 0.31**	0.33 ± 0.04
LY335979	3	0.18 ± 0.10*	1.22 ± 0.29**	0.02 ± 0.01
Verapamil	4	18.9 ± 4.2	0.84 ± 0.14	3.91 ± 1.78
Quinidine	4	33.8 ± 11.9	0.88 ± 0.14	10.8 ± 1.9**
Citalopram	3	n.d.	0.05 ± 0.03**	157.4 ± 56.8
Desmethylcitalopram	3	n.d.	0.04 ± 0.02**	133.5 ± 9.4**
Fluoxetine	4	115.5 ± 11.7**	0.08 ± 0.06**	91.4 ± 7.7**
Norfluoxetine	3	n.d.	0.08 ± 0.01**	59.0 ± 16.1**
Fluvoxamine	4	n.d.	0.07 ± 0.04**	64.0 ± 16.8**
Paroxetine	4	29.8 ± 11.1	0.37 ± 0.04**	14.3 ± 3.5
Paroxetine-M	3	n.d.	0.07 ± 0.03**	97.2 ± 31.2**
Reboxetine	4	n.d.	0.12 ± 0.03**	71.8 ± 17.8**
Sertraline	4	31.8 ± 2.8	0.83 ± 0.08	10.0 ± 0.9
Desmethylsertraline	3	n.d.	0.49 ± 0.22	23.1 ± 5.6
Venlafaxine	5	n.d.	0.01 ± 0.01**	n.d.
N-desmethyl-venlafaxine	3	n.d.	0 ± 0	440 ± 78
O-desmethyl-	2	n.d.	n.d.	n.d.

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venlafaxine				

n = number of experiments, each performed in octuplet. Values represent mean ± SD of at least three independent assays. P-values are determined by ANOVA with Dunnett's multiple comparison test for post hoc pairwise comparison of the results with the verapamil control. * p<0.05, **p<0.01, n.d. = not definable.

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Table 2: Inhibition of Pgp by newer antidepressants and typical Pgp inhibitors in pBCECs.

Test compound	n =	IC_{50} (µM)	IP_{50rel} (for SDZ-PSC833 $IP_{2.5rel}$ and for LY335979 IP_{1rel})	Concentration (µM) needed to double baseline fluorescence (f2)
SDZ-PSC833	3	0.02 ± 0.01**	0.98 ± 0.14	0.02 ± 0.01
LY335979	3	0.01 ± 0.002**	1.10 ± 0.33	0.003 ± 0.001
Verapamil	4	2.01 ± 0.04	1.09 ± 0.21	1.90 ± 1.53
Quinidine	3	3.16 ± 1.24	0.69 ± 0.12**	2.55 ± 1.59
Citalopram	4	n.d.	0.17 ± 0.03**	63.0 ± 12.9*
Desmethylcitalopram	4	n.d.	0.13 ± 0.03**	133.1 ± 29.1**
Fluoxetine	5	n.d.	0.14 ± 0.05**	n.d.
Norfluoxetine	4	n.d.	0.21 ± 0.05**	39.6 ± 2.9
Fluvoxamine	3	n.d.	0.23 ± 0.04**	51.8 ± 13.8
Paroxetine	3	n.d.	0.36 ± 0.04**	14.0 ± 5.3
Paroxetine-M	4	n.d.	0.21 ± 0.05**	47.5 ± 15.1
Reboxetine	3	n.d.	0.23 ± 0.01**	34.9 ± 4.1
Sertraline	3	n.d.	0.62 ± 0.14**	14.4 ± 9.3
Desmethylsertraline	5	n.d.	0.25 ± 0.11**	8.3 ± 4.3
Venlafaxine	4	n.d.	0.05 ± 0.03**	n.d.
N-desmethyl-venlafaxine	3	n.d.	0.01 ± 0.01	n.d.
O-desmethyl-venlafaxine	2	n.d.	n.d.	n.d.

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n = number of experiments, each performed in octuplet. Values represent mean ± SD of at least three independent assays. P-values are determined by ANOVA with Dunnett's multiple comparison test for post hoc pairwise comparison of the results with the verapamil control. * $p<0.05$, ** $p<0.01$, n.d. = not definable.

Figure legends:

Figure 1: Calcein assay. Evaluation of the inhibitory effect of a compound by calculation of the concentration required to increase baseline calcein fluorescence 2-fold (example: paroxetine in pBCECs, n = 8 wells).

Figure 2: Expression of human Pgp (hPgp) and porcine pgp1A (ppgp1A) at the mRNA level in L-MDR1, LLC-PK1 and pBCECs detected by RT-PCR. 1.5% agarose gel electrophoresis; lane 1, 6, 11: molecular size marker pUC19/*Msp*I; lane 2, 7: no template control; lane 3-5: detection of hPgp in L-MDR1 (3), LLC-PK1 (4) and pBCECs (5); lane 8-10: detection of ppgp1A in L-MDR1 (8), LLC-PK1 (9) and pBCECs (10).

Figure 3: Calcein-AM uptake in LLC-PK1 and L-MDR1 cells and influence of the Pgp inhibitor verapamil (VPL, 200 µM).

Data are given as means ± SD for n = 14 experiments performed in octuplet. P-values are determined by ANOVA with Dunnett's multiple comparison test for post hoc pairwise comparison of the results with the LLC-PK1 cells without inhibitor.

* Indicates significant difference compared to LLC-PK1 ($p<0.01$).

Figure 4: Calcein assay. Concentration dependent effect of control compounds (A) and the three antidepressants tested reaching a plateau (B) on the calcein accumulation in L-MDR1 cells. Each curve depicts one representative experiment of a series of 3-5. Data are expressed as mean ± SD for n = 8 wells.

Figure 5: Calcein accumulation in relation to verapamil in L-MDR1 cells. Values were calculated by dividing f2 of the control verapamil by f2 of the respective test compound. For

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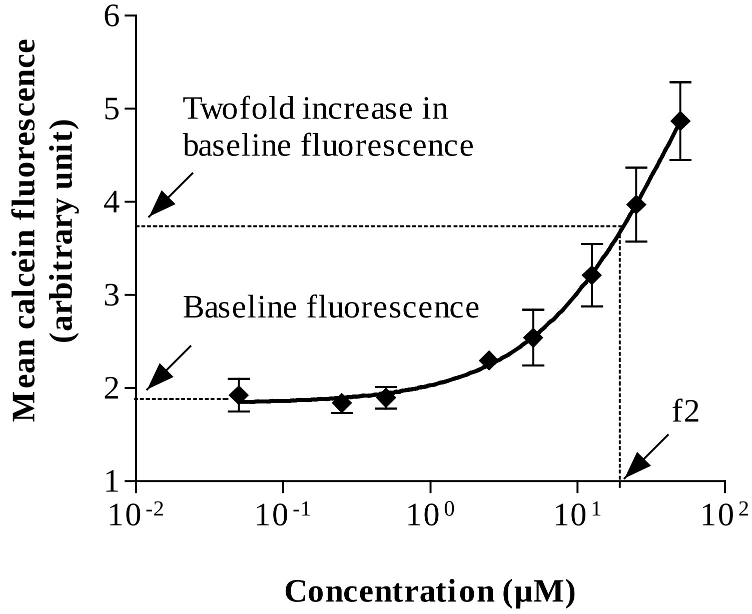
venlafaxine and O-desmethylvenlafaxine this value was not definable (see Table 2). Control compounds are drawn in black, antidepressants in gray. N-dm- = N-desmethyl-.

Figure 1



6
5
4
3
2
1
Mean calcein fluorescence
(arbitrary unit)
Twofold increase in
baseline fluorescence
Baseline fluorescence
f2
10 -2
10 -1
10 0
10 1
10 2
Concentration (µM)

Figure 2

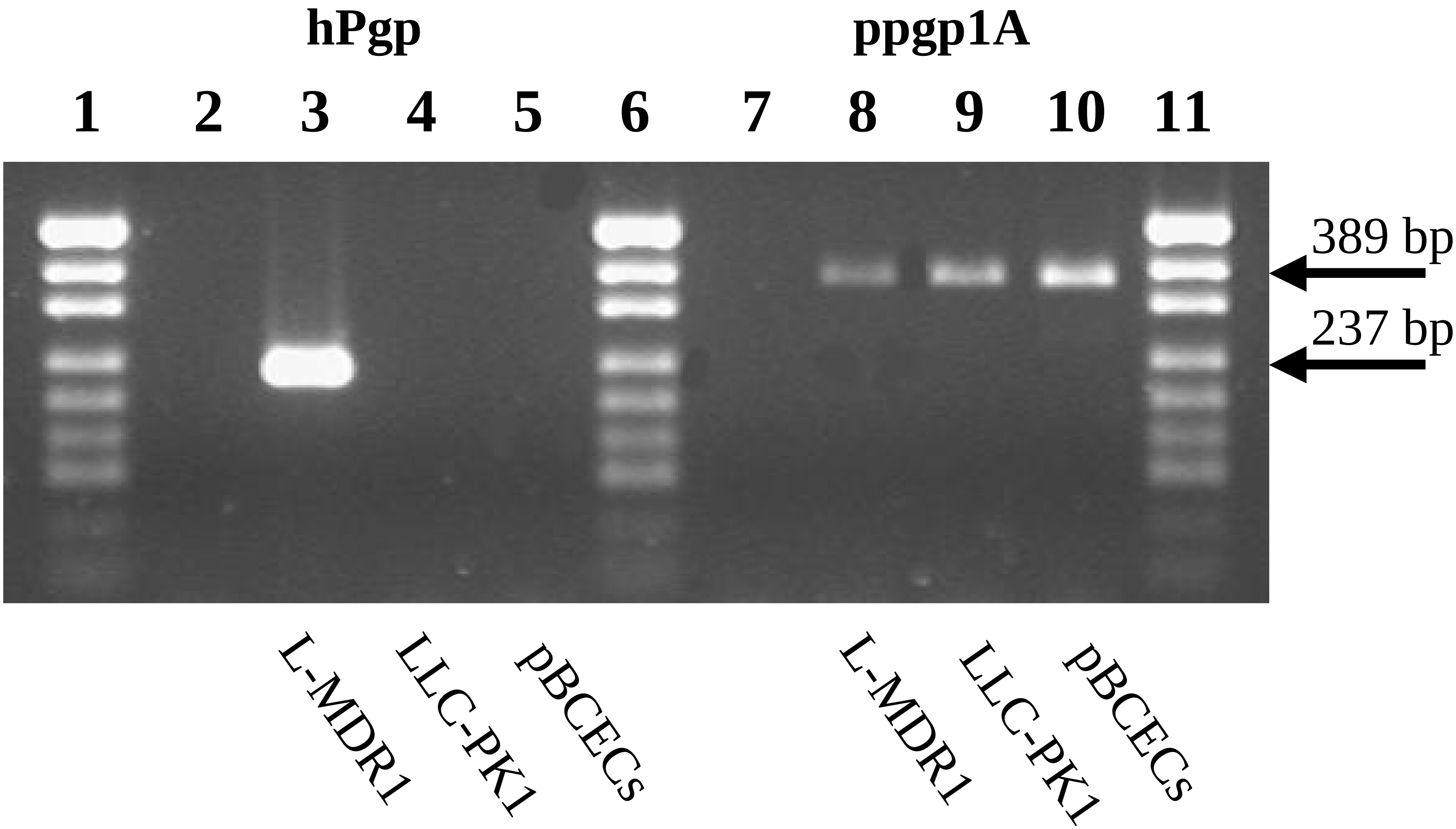


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Figure 3

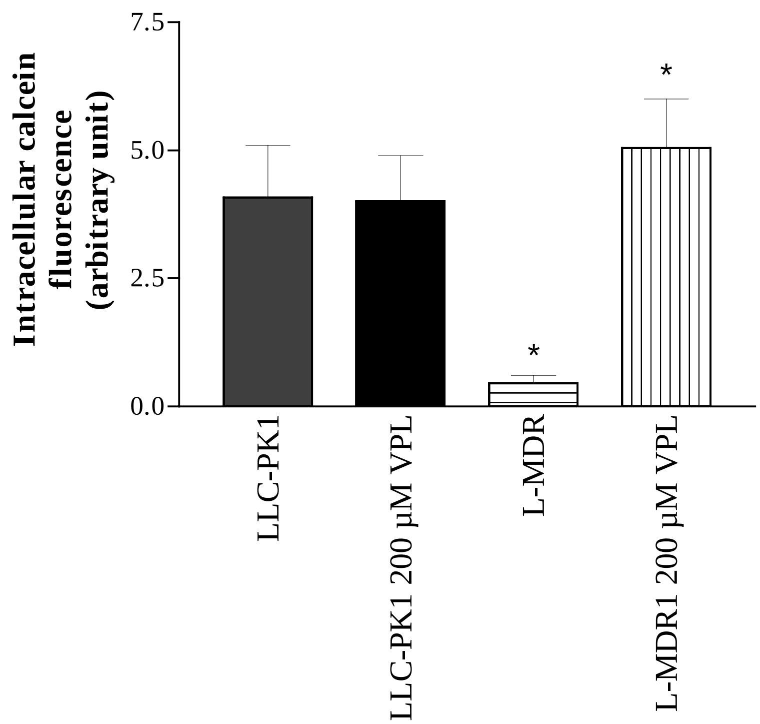

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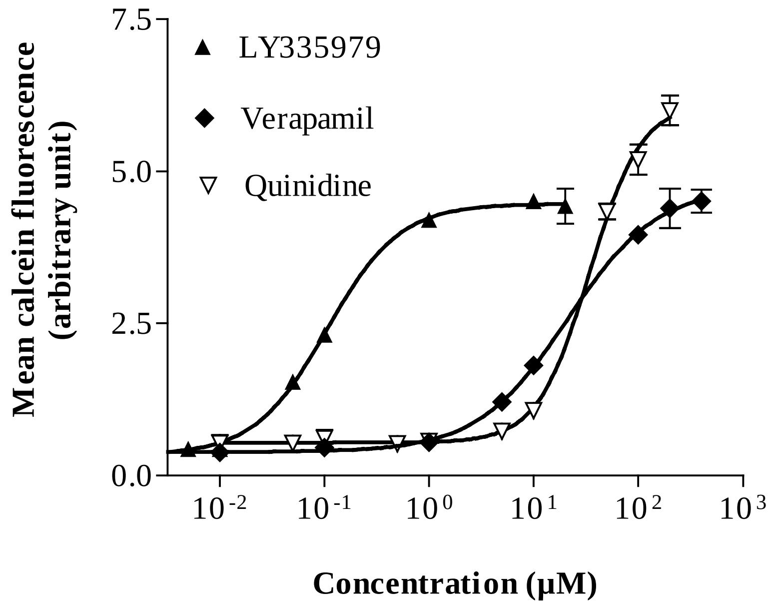
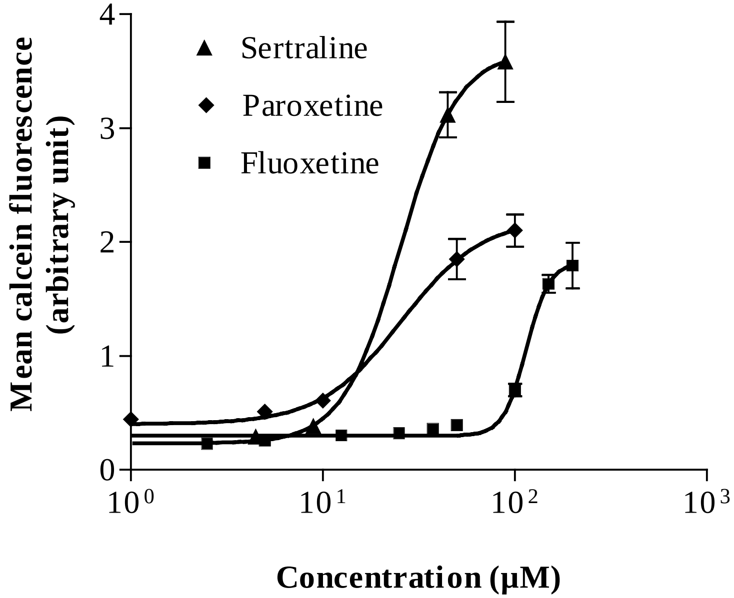
Figure 4a



7.5
5.0
2.5
0.0
LY335979
Verapamil
Quinidine
Mean calcein fluorescence (arbitrary unit)
10 -2
10 -1
10 0
10 1
10 2
10 3
Concentration (µM)

Figure 4b



Sertraline
Paroxetine
Fluoxetine
Mean calcein fluorescence (arbitrary unit)
Concentration (µM)
0
1
2
3
4
10⁰
10¹
10²
10³

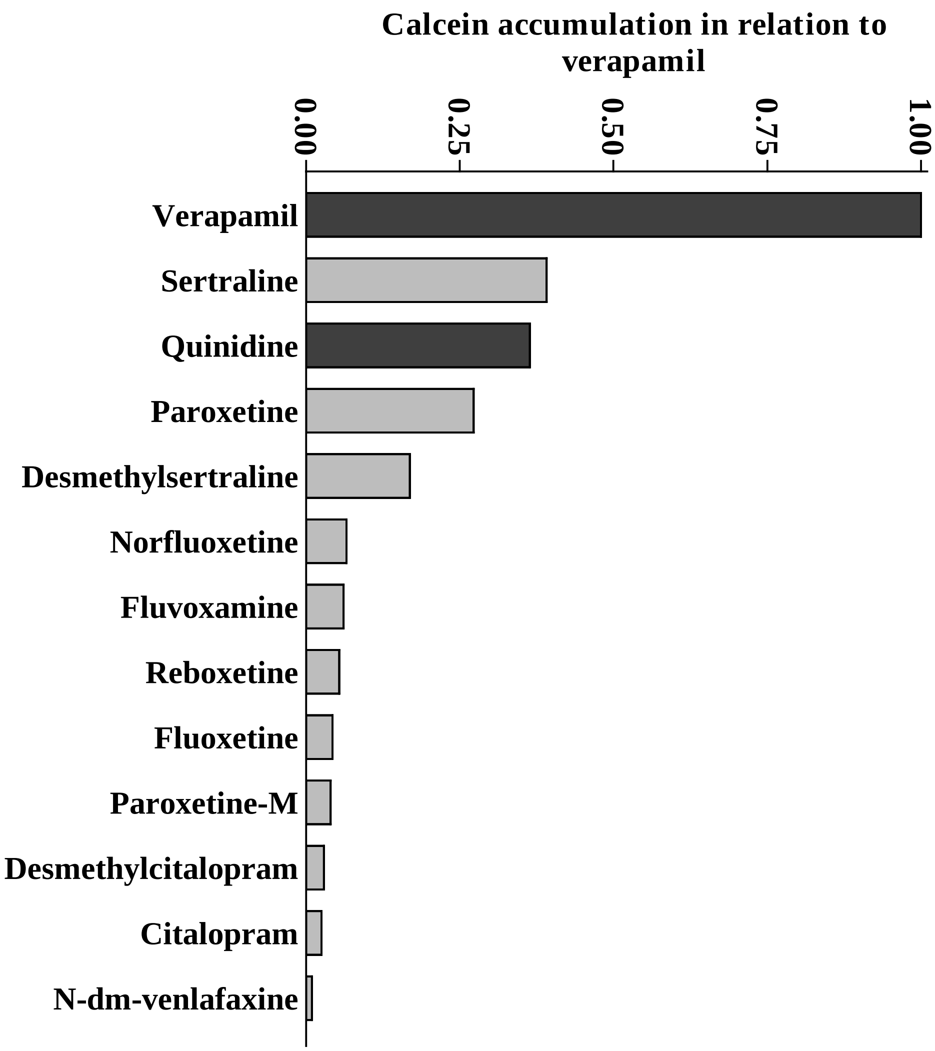


Figure 5

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