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Comparison of *in vitro* cell models in predicting *in vivo* brain entry of drugs

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ABSTRACT

Although several *in vitro* models have been reported to predict the ability of drug candidates to cross the blood–brain barrier, their real *in vivo* relevance has rarely been evaluated. The present study demonstrates the *in vivo* relevance of simple unidirectional permeability coefficient (P_{app}) determined in three *in vitro* cell models (BBMEC, Caco-2 and MDCKII-MDR1) for nine model drugs (alprenolol, atenolol, metoprolol, pindolol, entacapone, tolcapone, baclofen, midazolam and ondansetron) by using dual probe microdialysis in the rat brain and blood as an *in vivo* measure. There was a clear correlation between the P_{app} and the unbound brain/blood ratios determined by *in vivo* microdialysis (BBMEC $r = 0.99$, Caco-2 $r = 0.91$ and MDCKII-MDR1 $r = 0.85$). Despite of the substantial differences in the absolute *in vitro* P_{app} values and regardless of the method used (side-by-side vs. filter insert system), the capability of the *in vitro* models to rank order drugs was similar. By this approach, thus, the additional value offered by the true endothelial cell model (BBMEC) remains obscure. The present results also highlight the need of both *in vitro* as well as *in vivo* methods in characterization of blood–brain barrier passage of new drug candidates.

1. Introduction

The blood–brain barrier controls the access of both endogenous compounds and xenobiotics such as drugs into the central nervous system. The brain capillary endothelial cells with tight junctions effectively restrict the paracellular permeability of compounds. In addition, several active mechanisms such as carrier mediated influx and efflux transporters (*e.g.* P-glycoprotein; P-gp) control the passage of substances from the circulation into the central nervous system. The endothelial cells of blood–brain barrier can also metabolize drugs and thus prevent the penetration of drugs into the brain (Pardridge, 2003).

There is a need for reliable methods to characterize the pharmacokinetic properties of new drug candidates as early as possible to decrease the risk for failure during the later phases of the drug development process (Reichel, 2006). Therefore, many *in vitro*, *in vivo*, *in situ* and *in silico* methods for assessing the characteristics of new drug candidates are under evaluation (Feng, 2002). *In vitro* methods are commonly used for early estimation of pharmacokinetic characteristics of new drug candidates and to rank candidates for further stages of drug development process. This is usually conducted with high throughput and simple permeation experiments; the permeation characteristics of a new drug candidate can be approximated by unidirectional apparent permeability coefficient (P_{app}) values measured in the apical-to-basolateral (AB) direction (Abbott et al., 2008).

In vitro cell models such as human epithelial colorectal adenocarcinoma (Caco-2) or Madin-Darby canine kidney II cells transfected with the human multidrug resistance gene 1 (encoding P-gp) (MDCKII-MDR1) are commonly used to evaluate the blood–brain barrier permeability of drugs (Lundquist et al., 2002; Garberg et al., 2005; Wang et al., 2005). Primary brain endothelial cells isolated from the brain tissue are authentic blood–brain barrier cells and possess the closest similarity to the *in vivo* blood–brain barrier (Gumbleton and Audus, 2001). Thus, these cells may represent a more relevant, although more laborious, model of the blood–brain barrier than cells isolated from epithelial tissues. This hypothesis has been supported by *in vitro* data obtained with primary bovine brain microvessel endothelial cells (BBMEC), which have been suggested to be a good indicator for the ability of a drug to cross the blood–brain barrier *in vivo* (Eddy et al., 1997; Hansen et al., 2002; Lundquist et al., 2002).

Although *in vitro* cell models are routinely used in drug development, only a few papers have focused on evaluating their true *in vivo* relevance. Selection of the *in vivo* parameter as a counterpart for the *in vitro* parameter is crucial, since it determines the predictive applications of the *in vitro* parameter. Various methods have

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been used to assess the drug transport across the blood–brain barrier *in vivo*. For example, the permeability-surface area (PS) product determined with the *in situ* brain perfusion technique is a widely used parameter to assess the rate of drug transport into the brain (Smith, 2003; Hammarlund-Udenaes et al., 2008; Liu et al., 2008). The *in vivo* microdialysis method has proven useful in the characterization of the pharmacokinetic and pharmacodynamic properties of drugs (Chaurasia et al., 2007), and modifications of the method have been used in brain penetration studies, *e.g.* with atenolol and acetaminophen (de Lange et al., 1994), theophylline (Sjöberg et al., 1992), carbamazepine (Van Belle et al., 1995), baclofen (Deguchi et al., 1995), gabapentin (Wang and Welty, 1996), and oxycodone (Boström et al., 2006). The microdialysis technique allows continuous monitoring of unbound drug concentrations as a function of time simultaneously on both sides of the blood–brain barrier, *i.e.* in the brain extracellular fluid and in the blood, by inserting one probe into the brain tissue and another in the peripheral blood vessel (Hammarlund-Udenaes et al., 1997). Thus, microdialysis can be applied as a tool to explore drug equilibration across the blood–brain barrier by using the ratio of AUC in brain extracellular fluid to that in blood (Hammarlund-Udenaes et al., 1997; Chaurasia et al., 2007). This *in vivo* unbound brain/blood ratio determined by *in vivo* microdialysis not only describes the ability of a drug molecule to cross the blood–brain barrier but also takes into account other pharmacokinetic processes.

The general aim of the present study was to evaluate whether a simple *in vitro* parameter such as the unidirectional P_{app} AB can reliably predict the ability of a new drug candidate to cross the blood–brain barrier *in vivo* after a single intraperitoneal dose. Since the *in vitro* P_{app} often is routinely determined in the early stage of the drug development process, it is of interest to evaluate whether this value can be used to predict the *in vivo* fate of a new drug candidate. For this purpose, the *in vitro* permeabilities of the BBMEC, Caco-2 and MDCKII-MDR1 models for nine model drugs with different physicochemical characteristics were determined. The *in vivo* unbound brain/blood ratios for these model compounds were assessed using a dual probe microdialysis method. Then, the rank order of the model drugs obtained *in vivo* was compared to that determined *in vitro*, although it is accepted that the pharmacokinetic processes described by the unidirectional *in vitro* P_{app} and the *in vivo* unbound brain/blood ratios are fundamentally different. In addition, we wanted to find out whether the three *in vitro* models used differ in their *in vivo* relevance and whether the true brain endothelial cell model, the BBMEC model, offers additional value over the commonly used epithelial cell models.

2. Materials and methods

2.1. Drugs

Four β-blocking agents (alprenolol hydrochloride, atenolol, metoprolol tartrate, and pindolol), two catechol-*O*-methyltransferase (COMT; EC 2.1.1.6) inhibitors (entacapone and tolcapone), a 5-HT_3 antagonist (ondansetron hydrochloride dihydrate), a γ-aminobutyric acid analog (±-baclofen), and a benzodiazepine derivative (midazolam) were included into these studies. These nine model drugs were selected to cover a wide range of physicochemical properties and therapeutic targets. The main selection criteria were molecular weight between 200 and 400 Da and adequate hydrophilicity ($\log D < 4$) in order to ensure the suitability of the drugs for the microdialysis set-up. With respect to efflux transport, ondansetron is the only confirmed P-gp substrate of the model drugs (Schinkel et al., 1996). All drugs, except midazolam (Dormicum®, Roche, Basel, Switzerland), entacapone and tolcapone (Orion Pharma, Finland) were purchased from Sigma Chemicals (St. Louis, MO, USA). All drug concentrations and doses refer to the base form.

2.2. In vitro permeation studies

2.2.1. Cells

BBMECs were isolated based on the method described earlier (Audus and Borchardt, 1987; Audus et al., 1996). The isolated microvessel fragments were frozen under liquid nitrogen until used.

Caco-2 wild type cell line was obtained from American type culture collection (Manassas, VA, USA) and used between passages 45–49. The cells were maintained in Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin and passaged at 80–90% confluence with 0.25% Trypsin-0.53 mM EDTA. All Caco-2 cell culture materials were supplied by LGC Promochem (Teddington, UK).

MDCKII-MDR1 cell line was obtained from the Netherlands Cancer Institute, Amsterdam, and used between passages 33–59. Cells were maintained in Gibco Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (LGC Promochem), 100 IU/ml penicillin–100 μg/ml streptomycin (LGC Promochem) and passaged at 80–90% confluence with 0.05% Trypsin (BioWhittaker, Cambrex, East Rutherford, NJ, USA), 1 mM EDTA.

2.2.2. Drug solutions

The drug solutions were prepared daily at a concentration of 20 μM (alprenolol, pindolol, metoprolol, ondansetron, midazolam, baclofen, entacapone, and tolcapone) or 100 μM (atenolol) in buffer solution (129 mM NaCl, 0.63 mM $CaCl_2$, 2.5 mM KCl, 0.74 mM $MgSO_4 \cdot 7H_2O$, 7.4 mM $Na_2HPO_4 \cdot 2H_2O$, 1.3 mM KH_2PO_4, 5.3 mM D-glucose, and 0.1 mM ascorbic acid, pH 7.4) (Borges et al., 1994). The pH of the solutions was adjusted to 7.4 ± 0.05 before use, where applicable.

2.2.3. Permeation experiments

2.2.3.1. BBMEC. The microvessel fragments were thawed and plated on polycarbonate filter membranes (0.4 μm Nuclepore Track-Etch, Whatman, Brentford, Middlesex, UK) on petri dishes (Sarstedt AG & Co., Nümbrecht, Germany) coated with 0.43 mg/cm^2 collagen extracted in-house from rat tails as described earlier (Pasonen-Seppänen et al., 2001) and 4.8 μg/cm^2 fibronectin (Sigma Chemicals). The cultures were grown as described earlier (Audus and Borchardt, 1987) with minor modifications. The culture medium contained 45% Gibco minimal essential medium (Invitrogen), 45% Gibco Ham's F-12 nutrient mix (Invitrogen), and 10% plasma-derived horse serum supplemented with 10 mM Hepes (pH 7.4), 13 mM sodium bicarbonate, 100 μg/ml penicillin G, 100 μg/ml streptomycin, 150 μg/ml heparin, 50 μg/ml polymyxin B, and 2.5 μg/ml amphotericin B, all supplied by Sigma Chemicals. The cultures were grown for three days at 37 °C in a humidified atmosphere at 5% CO_2. Thereafter, the cultures were grown in culture medium supplemented with 50 μg/ml heparin and 20 μg/ml bovine endothelial cell growth factor (Roche) without polymyxin B and amphotericin B. The medium was changed every two to three days until the cells were confluent when examined alongside polycarbonate membranes by visual inspection with a phase contrast microscope, and the cells together with the underlying filters were transferred into side-by-side diffusion chambers (PermeGear, Inc., Bethlehem PA, USA) (Fig. 1A). The permeation experiments were conducted as described by Borges et al. (1994) in an apical-to-basolateral direction at 37 °C. At the beginning of the experiment, the drug solution was introduced into the donor chamber (3 ml volume) and pure buffer solution was added to the receiver chamber

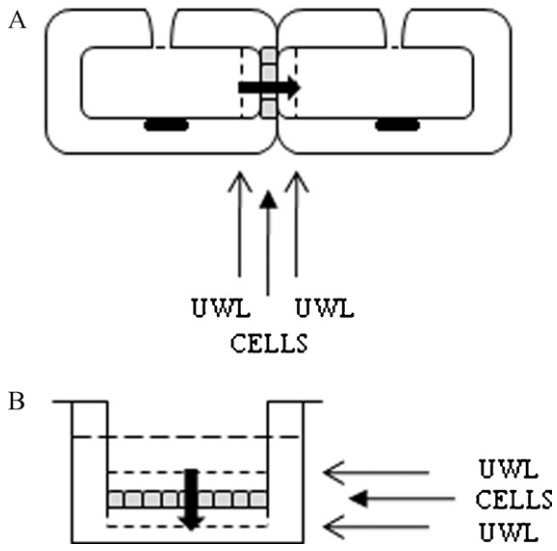

Fig. 1. Schematic illustration of the side-by-side diffusion chamber system (A) and the filter insert system (B) used in the present study. The cell monolayer (cells) and unstirred water layer (UWL) are indicated with narrow arrows. The apical-to-basolateral direction of the drug transport is demonstrated with bold arrow.

(3 ml volume). Constant magnetic stirring was used in both chambers. Samples were withdrawn at 5, 10, 20, 30, 45, and 60 min and fresh buffer was used to replace the fluid loss from the receiver chamber.

The integrity of the cell monolayer was analyzed by the permeation of [^{14}C]sucrose as a low permeability reference compound (0.1 μCi/ml; American Radiolabeled Chemicals, St. Louis, MO, USA). [^{3}H]diazepam was used as a high permeability reference compound (1 μCi/ml; PerkinElmer Life Sciences, Boston, MA, USA). All permeation experiments were conducted in triplicate by using at least three different batches of cells. The blank filter permeabilities were studied as described above but without the cell monolayers. The samples were stored at −80 °C until analyzed.

2.2.3.2. Caco-2 and MDCKII-MDR1. The Caco-2 and MDCKII-MDR1 permeation studies, were performed in Transwell® permeable supports (0.4 μm pore size polycarbonate 12-well, Costar, Corning, NY, USA). Briefly, Caco-2 cells were seeded at a density of 82×10^3 cells/cm^2 and MDCKII-MDR1 cells 39×10^4 cells/cm^2 onto 12-well Transwell®. Caco-2 medium was changed three times a week and the cells were allowed to grow for 21 days. MDCKII-MDR1 medium was changed daily and the cells were allowed to grow for 4 days. The permeation experiments were conducted on Transwell® (Fig. 1B) in an apical-to-basolateral direction at 37 °C by using Hank's balanced salt solution (Biowhittaker) buffered with 25 mM Hepes, 0.02% sodium chloride (Biowhittaker) (pH 7.4) in an orbital shaker (Titramax 1000, Heidolph, Germany) at 400 rpm. At the beginning of the experiment, the drug solution was introduced into the donor chamber (0.5 ml) and pure buffer solution was added to the receiver chamber (1.5 ml). Samples were withdrawn at 15, 30, 60, 120, and 180 min and fresh buffer was used to replace the fluid loss from the receiver chamber. The samples from the donor chamber were taken at 0 and 180 min.

The integrity of the cell monolayer was analyzed by the permeation of [^{14}C]sucrose as a low permeability reference compound (2 μCi/ml; American Radiolabeled Chemicals). [^{14}C]diazepam was used as a high permeability reference compound (0.1 μCi/ml; Amersham Biosciences, Buckinghamshire, UK). All permeation experiments were conducted in triplicate by using at least three different batches of cells. The blank filter permeabilities were studied as described above but without the cell monolayers. The samples were stored at −80 °C until analyzed.

2.3. In vivo microdialysis

2.3.1. Animals

Male Han/Wistar rats, supplied by the National Laboratory Animal Centre (Kuopio, Finland) were housed in stainless steel cages and kept on a 12-h light/12-h dark cycle at an ambient temperature. The animals were 8 weeks old and weighed approximately 250 g at the beginning of the studies ($n = 65$). Animals had free access to pelleted food (Lactamin R36; Lactamin AB, Södertälje, Sweden) and fresh tap water. All procedures with the animals were performed according to appropriate European Community Guidelines and reviewed by the Animal Ethics Committee at the University of Kuopio, and approved by the local provincial government.

2.3.2. Drug solutions

Metoprolol, alprenolol and baclofen were dissolved in Krebs Ringer solution (138 mM NaCl, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 11 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \cdot H_2O$, and 11 mM D-Glucose, pH 7.4) (Benveniste and Hüttemeier, 1990). Atenolol and pindolol were first dissolved in 0.1 M hydrochloric acid (10% of final volume) and diluted with Krebs Ringer solution. Entacapone and tolcapone were first dissolved in dimethyl sulfoxide (2% of final volume) and diluted to a final concentration with 10 mM phosphate buffer (pH 7.4). Commercial midazolam solution was diluted with saline to 10 μmol/ml and ondansetron was dissolved in saline. Drugs were administered i.p. at a dose of 50 μmol/kg in a volume of 5 ml/kg (entacapone and tolcapone 10 ml/kg). The recovery of the microdialysis probes was determined by preparing 1 mM stock solutions as described above and subsequently diluted to 1 μM with Krebs Ringer solution.

2.3.3. Microdialysis

All surgical procedures and microdialysis were performed under chloral hydrate anaesthesia (350 mg/kg i.p.; Sigma Chemicals). A single subcutaneous dose of buprenorfine (0.02 mg/kg; Schering-Plough, Belgium) was given after guide cannula implantation to relieve postoperative pain. A low molecular weight heparin (dalteparin 20 IU/ml, Fragmin®, Pfizer Health AB, Stockholm, Sweden) was added to the venous probe perfusate to prevent blood clotting around the probe membrane. To minimize tissue damage during the microdialysis experiment, an intracerebral guide cannula (MAB 6.10.IC, AgnTho's AB, Lidingö, Sweden) was implanted stereotaxically into the striatum (coordinates from bregma: AP +0.5 mm; L −3.0 mm; DV −3.8 mm) and fixed to the skull with anchor screws and dental cement one week before the microdialysis experiment. On the day of the microdialysis experiment, the rat was anesthetized and the i.v. microdialysis probe (MAB 11.20.10; 10 mm exposed membrane, 6 kDa cut-off, AgnTho's AB) was inserted into the left femoral vein. The animal was placed in a stereotaxic frame, and the central nervous system probe (MAB 9.10.4; 4 mm exposed membrane, 6 kDa cut-off, AgnTho's AB) was inserted slowly into the striatum through the guide cannula, the tip of the probe extending to −7.8 mm ventrally from the surface of the skull.

Both probes were perfused with Krebs Ringer solution at a flow rate of 2 μl/min. The probes were perfused for 80 min before drug administration in order to ensure the recovery of the integrity of the blood–brain barrier after probe insertion. Slow implantation of the probe has been shown to reduce the brain trauma (Allen et al., 1992). It has also been reported that the blood–brain barrier around the microdialysis probe is intact shortly (30 min–2 h) after the probe implantation (Benveniste and Hüttemeier, 1990). Dialysate was collected in twenty-minute fractions for 5 h into 300 μl polypropylene vials (AgnTho's AB) with a fraction collec-

tor. The samples were frozen at −20 °C and stored at −80 °C until analyzed.

The probe calibration was performed *in vitro* (de Lange et al., 1994). The *in vitro* recovery was determined as the ratio of a drug in the dialysate to the drug concentration (1 μM) in a non-stirred bulk Krebs Ringer solution at 37 °C (C_{dial}/C_{bulk}; 3 probes for each drug, 3 determinations per probe; flow rate 2 μl/min, collection time 20 min).

2.4. Drug analysis

New analytical methods were set up for alprenolol, atenolol, pindolol, entacapone, tolcapone, baclofen, midazolam and ondansetron.

2.4.1. Alprenolol, pindolol, entacapone and tolcapone

Alprenolol, pindolol, entacapone and tolcapone were analyzed by using liquid chromatography–mass spectrometry (LC–MS) with reversed-phase chromatographic separation and MS/MS detection. Two sets of instrument conditions were developed, one for the analysis of the alprenolol and pindolol, and the other for tolcapone and entacapone. The instrumentation consisted of a Surveyor high-performance liquid chromatography (HPLC) system (Thermo Scientific, San Jose, CA, USA) with a Zorbax XDB C18 column (2.1 × 100 mm, 3.5 μm; Agilent Technologies, Palo Alto, CA, USA). The MS analysis was carried out with LTQ linear ion trap mass spectrometer equipped with an electrospray ionization source operating in the positive ion mode (Thermo Scientific). For each analyte, separate ten-point calibration curves were prepared using appropriate buffer solution from *in vitro* experiments or *in vivo* microdialysis. In addition to standards, the sequences included blank and zero samples and quality control samples at four concentration levels. For quantification, peak area ratios of the analyte and the internal standard were calculated as a function of the concentration of the analyte using Thermo LCquan 2.0 quantification software.

For alprenolol and pindolol, chromatographic separations were performed using gradient elution with (A) water (Milli-Q Gradient system, Millipore, Milford, MA, USA) and (B) acetonitrile (J.T Baker HPLC Ultra Gradient Grade; Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA), both containing 0.1% (v/v) of formic acid (Sigma–Aldrich, St. Louis, MO, USA), as follows: 0–2.5 min: 15% B → 40% B; 2.5–3.5 min: 40% B; 3.5–3.6 min: 40% B → 15% B; 3.6–5.5 min: 15% B. Flow rate was 0.3 ml/min, column temperature 30 °C and autosampler tray temperature 5 °C. The injection volume was 15 μl. The following ionization conditions were used: nitrogen sheath, auxiliary and sweep gas flow rates 50, 10 and 10 instrument units, respectively and spray voltage 4.5 kV. Analyte detection was performed using the following transitions: m/z 250 → 116 for alprenolol, m/z 249 → 116 for pindolol, and 329 → 311 for labetalol (used as an internal standard). Collision energy was set to 40% for alprenolol and pindolol and 30% for labetalol. The divert valve was programmed to allow eluent flow into the mass spectrometer from 1.5 to 5.0 min of each 5.5 min run.

For entacapone and tolcapone, a 3 min isocratic run using 50% acetonitrile with 0.1% (v/v) formic acid was used. The flow rate was 0.3 ml/min, column temperature 30 °C, autosampler tray temperature 10 °C and injection volume 10 μl. Ionization conditions were the same as stated above. Analyte detection was performed using the following transitions: m/z 306 → 233 for entacapone and m/z 274 → 182 for tolcapone, both with collision energy set to 30%. For the analysis of entacapone, tolcapone was used as an internal standard, and *vice versa*. The divert valve was programmed to allow eluent flow into the mass spectrometer from 1 min onwards.

2.4.2. Atenolol

Atenolol was analyzed by HPLC (Agilent Technologies HPLC 1100 system and Agilent Technologies Chemsation for LC 3D Software Rev. A.08.03). Mobile phase A consisted of 0.05 M potassium dihydrogen phosphate and 0.01 M 1-octanesulfonate (both from Sigma–Aldrich). The pH was adjusted to 3.0 with hydrochloric acid and degassed for 15 min. Methanol was used as mobile phase B. The isocratic elution was performed by mixing A and B 62:38 (v/v). A Zorbax SB-phenyl-column (2.1 × 100 mm, 3.5 μm; Agilent Technologies) was used for chromatographic separation with a flow rate of 0.4 ml/min. The column was maintained at 40 °C. The injection volume was 10 μl and run-time 9 min. Atenolol was detected with a fluorescence detector at excitation wavelength of 230 nm and emission wavelength of 310 nm.

2.4.3. Ondansetron, midazolam and baclofen

Ondansetron, midazolam and baclofen were analyzed by LC–MS with reversed-phase chromatographic separation. Waters Acquity ultra performance liquid chromatograph (UPLC, Waters Corp., Milford, MA, USA) system consisted of sample organiser, column oven and vacuum degasser, and a Waters BEH C18 column (2.1 × 50 mm, 1.7 μm) with an on-line precolumn filter. The data was acquired using a Waters Quattro Premier triple quadrupole mass spectrometer equipped with a Z-spray electrospray source, using multiple reaction monitoring mode detection. A positive ion polarity was used for all study compounds. For all compounds, a capillary energy of 3 kV, desolvation temperature of 350 °C and source temperature of 150 °C were used, the collision gas was argon at 4.0×10^{-3} mbar pressure, and nitrogen were used as desolvation and nebuliser gases. Cone voltages used were 36 V for midazolam, 16 V for baclofen and 30 V for ondansetron. The multiple reaction monitoring transitions were m/z 326 → 291 (collision energy 26 eV) for midazolam, m/z 214 → 116 and m/z 214 → 151 (30 and 16 eV) for baclofen, and m/z 295 → 170 and m/z 295 → 184 (23 and 27 eV) for ondansetron. For midazolam, a linear gradient elution from 95% (A) 2 mM ammonium acetate (BDH Laboratory Supplies, Poole, England): 5% (B) acetonitrile (Lichrosolv HPLC Gradient Grade from Merck, Darmstadt, Germany) to 40% B in 1.5 min and then to 70% B in 0.5 min was employed, followed by 0.2 min isocratic elution with 70% B and column equilibration. For baclofen, linear gradient elution from 95% (A) 0.1% formic acid (BDH Laboratory Supplies): 5% (B) acetonitrile to 10% B in 1.5 min and then to 70% B in 0.5 min was used, followed by column equilibration. For ondansetron, a linear gradient elution from 95% (A) 10 mM ammonia (BDH Laboratory Supplies): 5% (B) acetonitrile to 30% B in 1.5 min and then to 70% B in 0.5 min was employed, followed by 0.2 min isocratic elution with 70% B and column equilibration. The MS and HPLC systems were operated under Masslynx 4.1 software (Waters Corp.). The first 1 min of the run was directed into waste by using the divert valve in order to decrease the ion source contamination by early eluting matrix constituents.

2.4.4. Metoprolol

Metoprolol was analyzed with the HPLC method of Palmgrén et al. (2004). The following modifications were made; pick up injection (25 μl) with the 200 μl sample loop and fluorescence detection.

2.4.5. Validation of analytical methods

Analytical methods were partially validated with regard to specificity, selectivity, linearity, precision and accuracy based on the Food and Drug Administration guideline (Guidance for Industry, Bioanalytical Method Validation) (data not shown). Calibration ranges for the analytical methods are shown in Table 1.

Table 1
Calibration ranges for *in vivo* and *in vitro* samples for analytical methods.

Calibration range (nM)	*In vitro* samples	*In vivo* samples
Alprenolol	25–7000	1–250
Atenolol	2–3000	2–3000
Metoprolol	10–3000	10–3000
Pindolol	25–7000	1–250
Entacapone	1–800	1–400
Tolcapone	1–800	1–400
Baclofen	1–5000	0.5–1000
Midazolam	5–5000	0.1–1000
Ondansetron	5–10000	0.1–1000

2.4.6. Radiotracer samples

Radiotracer samples were analyzed by liquid scintillation counting (1450 MicroBeta Trilux Liquid Scintillation and Luminescence Counter, Wallac, Finland) after the addition of 500 μl scintillation cocktail Optiphase, Wallac (Milton Keynes, UK).

2.5. Data analysis

The apparent permeability coefficient (P_{app}, cm/s) for the drugs was calculated assuming steady state conditions according to Eq. (1):

$$P_{app} = \frac{\Delta Qr/\Delta t}{A \times C_d} \quad (1)$$

where $\Delta Qr/\Delta t$ is the steady state flux of drug, *i.e.*, the slope of the linear region of the cumulative amount of drug in receiver chamber vs. time (h) plot; C_d is the drug concentration in the donor chamber; and A is the membrane surface area (cm^2).

Microdialysate drug concentrations were corrected with recovery values. The $AUC_{0-\infty}$ values were calculated from individual data with the trapezoidal rule using GraphPad Prism 4.03 software (GraphPad Software, San Diego, CA, USA). The unbound brain/blood ratio *in vivo* was defined according to Eq. (2):

$$\text{brain/blood ratio} = \frac{AUC_{ECF_{0-\infty}}}{AUC_{blood_{0-\infty}}} \quad (2)$$

where AUC_{ECF} is the area under the concentration–time curve in brain extracellular fluid and AUC_{blood} is the area under the concentration–time curve in blood. The apparent elimination half lives in blood and in brain ECF ($t_{1/2\beta}$) were calculated from individual data using WinNonlin Professional v5.0.1 software (Pharsight Corporation, Mountain View, CA, USA).

In order to study the differences between the cell models, pairwise linear regressions were calculated using GraphPad Prism 4.03 software. The relationship between P_{app} values was analyzed with two-tailed Pearson correlation coefficients (r).

To evaluate the *in vivo* relevance of the P_{app} values determined in the cell models, each measured *in vitro* P_{app} value ($P_{app} \times 10^5$ in BBMEC and $P_{app} \times 10^6$ in Caco-2 and MDCKII-MDR1) was log-normalized, and a linear regression between *in vitro* $\log(P_{app})$ and brain/blood ratio *in vivo* was estimated, and two-tailed Pearson correlation coefficients were determined. All correlations were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. In vitro permeabilities

The obtained apical-to-basolateral P_{app} values are shown in Table 2. Major differences in P_{app} values of model drugs between different cell models were found. In the BBMEC cell model, the P_{app} value for the low permeability reference compound, sucrose was $34.9 \pm 13.5 \times 10^{-6}$ cm/s (mean ± S.D.) ($n = 30$) and for the high permeability reference compound, diazepam $374.3 \pm 62.5 \times 10^{-6}$ cm/s ($n = 30$). In the Caco-2 epithelial cell model, the P_{app} value for sucrose was $2.4 \pm 1.4 \times 10^{-6}$ cm/s ($n = 12$) and for diazepam $66.9 \pm 22.6 \times 10^{-6}$ cm/s ($n = 15$), and in the MDCKII-MDR1 cell model $0.7 \pm 0.3 \times 10^{-6}$ cm/s ($n = 18$) and $64.6 \pm 18.2 \times 10^{-6}$ cm/s ($n = 17$) for sucrose and diazepam, respectively.

Table 2
The apparent permeability coefficient (P_{app}, cm/s) values ($\times 10^6$) apical-to-basolateral direction for BBMEC, Caco-2 and MDCKII-MDR1 *in vitro* cell models. Data are mean ± S.D.

$P_{app} \times 10^6$ (cm/s)	BBMEC	Caco-2	MDCKII-MDR1
Alprenolol	437.6 ± 52.6 ($n = 9$)	52.3 ± 4.3 ($n = 8$)	53.4 ± 7.3 ($n = 9$)
Atenolol	48.5 ± 23.4 ($n = 9$)	0.9 ± 0.2 ($n = 9$)	0.7 ± 0.2 ($n = 8$)
Metoprolol	240.3 ± 62.7 ($n = 8$)	54.8 ± 6.9 ($n = 9$)	63.1 ± 14.5 ($n = 9$)
Pindolol	84.4 ± 15.5 ($n = 9$)	28.7 ± 4.3 ($n = 8$)	24.4 ± 3.6 ($n = 9$)
Entacapone	31.2 ± 13.6 ($n = 12$)	2.5 ± 0.6 ($n = 6$)	10.7 ± 2.9 ($n = 9$)
Tolcapone	444.8 ± 98.8 ($n = 9$)	63.2 ± 12.2 ($n = 9$)	63.5 ± 16.8 ($n = 9$)
Baclofen	35.9 ± 14.1 ($n = 12$)	0.9 ± 0.7 ($n = 8$)	0.7 ± 0.4 ($n = 8$)
Midazolam	294.8 ± 79.2 ($n = 12$)	39.3 ± 5.8 ($n = 9$)	42.0 ± 5.6 ($n = 8$)
Ondansetron	276.3 ± 27.2 ($n = 11$)	47.1 ± 5.2 ($n = 9$)	37.6 ± 3.9 ($n = 9$)

With this set of model drugs, the blank filter permeability ranged between 320–500 $\times 10^{-6}$ cm/s and 50–160 $\times 10^{-6}$ cm/s in side-by-side diffusion chambers and in the filter insert system, respectively. It seems that permeability values higher than 160×10^{-6} cm/s cannot be achieved in the filter insert system due to its limited stirring action. In contrast, the blank filter permeability values can be as high as 500×10^{-6} cm/s in side-by-side diffusion chambers.

3.2. In vivo microdialysis

The time-concentration profiles for the model drugs are presented in Fig. 2. All drugs studied were quantifiable in the brain extracellular fluid. The observed *in vivo* parameters calculated from microdialysis data and probe recoveries are shown in Table 3. The unbound brain/blood ratio was highest for alprenolol (0.77 ± 0.15) (mean ± S.D.) and lowest for baclofen (0.08 ± 0.10). Atenolol had the longest elimination half life in brain extracellular fluid (198 min), whereas the fastest elimination from brain extracellular fluid was determined for entacapone (29 min). In blood, the $t_{1/2\beta}$ varied between 39 (entacapone) and 125 min (baclofen). The recovery values for the brain probes varied between 10% and 29% for entacapone and pindolol, respectively. For blood probes, the highest recovery was determined for pindolol (50%), and the lowest for midazolam (18%).

3.3. Comparison of cell models

The drugs were divided into low, medium and high permeability categories as described earlier; in the BBMEC, the high permeability limit was set to 70×10^{-6} cm/s, and the low permeability limit to 30×10^{-6} cm/s (Eddy et al., 1997). In the Caco-2 and MDCKII-MDR1 models, the high permeability limit was $\sim 40 \times 10^{-6}$ cm/s, and the low permeability limit $\sim 18 \times 10^{-6}$ cm/s (Polli et al., 2000). In order to determine whether the models categorize the drugs into same permeability categories, the P_{app} values obtained in each model were compared by pairwise linear regressions (Fig. 3). The correlation between BBMEC and Caco-2 was $r = 0.93$, BBMEC and MDCKII-MDR1 $r = 0.91$, and Caco-2 and MDCKII-MDR1 $r = 0.98$. All correlations were statistically significant ($P < 0.001$). An excellent correlation was found between the epithelial cell models Caco-2 and MDCKII-MDR1 and they classify the drugs in the same permeability categories with the exception of the P-gp substrate ondansetron, which had a statistically significantly lower permeability in P-gp over-expressing MDCKII-MDR1 model than in Caco-2 model (37.6×10^{-6} cm/s vs. 47.1×10^{-6} cm/s) ($P < 0.001$, Student's t-test).

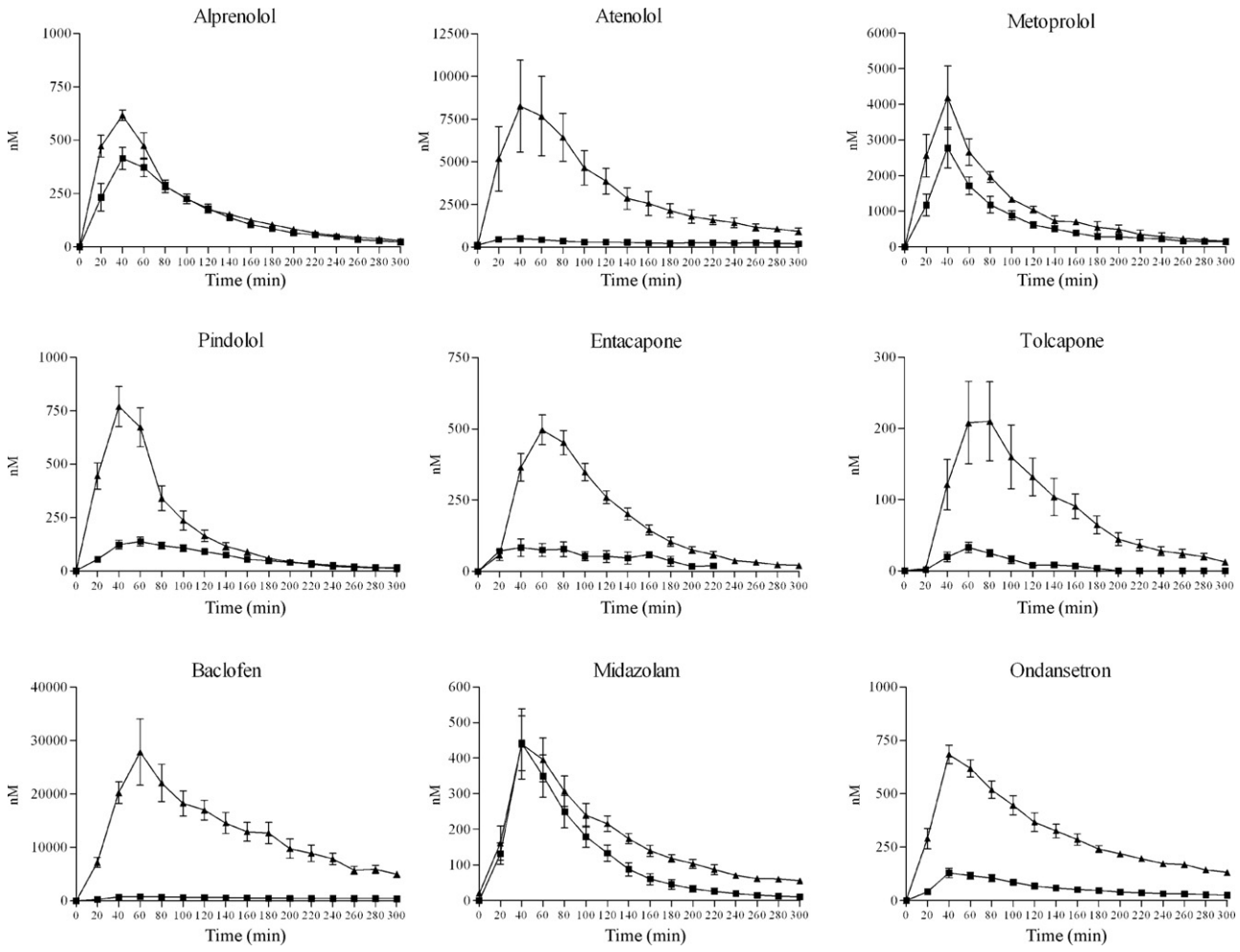

Fig. 2. Time–concentration profiles for the nine model drugs in the *in vivo* microdialysis study. Brain (■) and blood (▲) unbound drug concentrations represent group average ± S.E.M. (n = 5–10/drug). Drugs were administered i.p. at a dose of 50 μmol/kg. Note differences in the scales of the ordinate axis.

3.4. In vivo relevance of in vitro cell models

The *in vivo* brain/blood ratios are presented as a function of log-normalized *in vitro* P_{app} values in Fig. 4. When all drugs were included in the analysis, the correlations were statistically not significant (r = 0.62, r = 0.61 and r = 0.60 in BBMEC, Caco-2, and MDCKII-MDR1, respectively). After the exclusion of ondansetron and tolcapone, which were considered as outliers (see Discussion), the best correlation was found with the BBMEC model (r = 0.99, P < 0.001), followed by the Caco-2 (r = 0.91, P < 0.01) and MDCKII-MDR1 (r = 0.85, P < 0.05) models.

In order to examine the effect of filters, the permeability exclusively through the cell monolayer ($P_{(cell)}$, data not shown) was determined by subtracting the blank filter data from the total permeability. The *in vitro–in vivo* correlation coefficients remained similar (r = 0.98, r = 0.93, and r = 0.88 for BBMEC, Caco-2 and MDCKII-MDR1, respectively).

3.5. Rank order of model drugs

The rank order of the drugs from the highest to the lowest probability to enter the brain in each model (*i.e.,* BBMEC, Caco-2, MDCKII-MDR1 and *in vivo*) is illustrated in Table 4. Only minor differences in the rank order of the drugs between the cell models were found. The clearest difference between the cell models and the *in vivo* model was found with tolcapone; all *in vitro* models ranked it as a very high permeability compound, whereas in the *in vivo* model it was characterized by its low ability to enter into the brain.

4. Discussion

In the present study, the *in vitro* permeation characteristics of nine model drugs were studied in BBMEC, Caco-2 and MDCKII-MDR1 cell models. The P_{app} values obtained in each model were correlated with each other to characterize the differences between the models. To evaluate whether the simple P_{app} value is able to predict how well the drug is transferred across the blood–brain barrier, the *in vitro* P_{app} values of nine model drugs were correlated with *in vivo* unbound brain/blood ratios determined by a dual probe microdialysis set-up.

The determined *in vitro* P_{app} values were found to be clearly different in the three *in vitro* cell models evaluated, but still in agreement with the literature. For example, the P_{app} values for the paracellular marker sucrose were found to be similar as reported earlier in the Caco-2 and MDCKII-MDR1 (Garberg et al., 2005) and in the BBMEC model in well-stirred systems (Eddy et al., 1997).

In the BBMEC model, alprenolol, midazolam, metoprolol, pindolol, ondansetron, tolcapone and the high permeability reference compound diazepam were classified as drugs with high permeability. In the Caco-2 and MDCKII-MDR1 models, the high permeability

Table 3
Brain/blood ratios and $t_{1/2\beta}$ values, probe recovery values and physicochemical properties of the model drugs. Data are mean ± S.D. Brain/blood ratios and $t_{1/2\beta}$ values were determined using dual probe *in vivo* microdialysis data. Drugs were administered i.p. at a dose of 50 μmol/kg.

	Alprenolol		Atenolol		Metoprolol		Pindolol		Entacapone		Tolcapone		Baclofen		Midazolam		Ondansetron	
Brain/blood ratio	0.77 ± 0.15 (*n* = 9)		0.16 ± 0.08 (*n* = 5)		0.64 ± 0.06 (*n* = 6)		0.34 ± 0.08 (*n* = 8)		0.14 ± 0.11 (*n* = 10)		0.17 ± 0.12 (*n* = 6)		0.08 ± 0.10 (*n* = 9)		0.61 ± 0.19 (*n* = 6)		0.19 ± 0.07 (*n* = 6)	
Mol. weight	249.35		266.34		267.36		248.32		305.29		273.24		213.66		325.77		293.36	
Plasma protein binding (%)[a]	80[b]		3[b]		12[b]		60[b]		98[c]		99.9[c]		30[c]		96[c]		70[c]	
$\log D_{pH\ 7.4}$[d]	0.81		−1.76		−0.47		−0.5		0.0946		0.76		−1.72		3.78		1.2	
	Bl	Br	Bl	Br	Bl	Br	Bl	Br	Bl	Br	Bl	Br	Bl	Br	Bl	Br	Bl	Br
Recovery (%)[e]	37 ± 0	26 ± 0	29 ± 7	15 ± 4	32 ± 11	13 ± 1	50 ± 9	29 ± 4	31 ± 9	10 ± 1	24 ± 11	14 ± 9	32 ± 12	22 ± 3	18 ± 3	22 ± 10	45 ± 6	23 ± 10
$t_{1/2\beta}$ (min)[f]	66 ± 6	61 ± 7	90 ± 9	198 ± 53	57 ± 10	63 ± 8	49 ± 14	74 ± 11	39 ± 12	29 ± 9	60 ± 8	57 ± 30	125 ± 56	178 ± 136	91 ± 27	47 ± 16	106 ± 12	106 ± 26

$t_{1/2\beta}$ elimination half life; Bl = blood, Br = brain.
[a] Human data.
[b] Borchard (1998).
[c] European Medicines Agency.
[d] Computational values calculated by the Advanced Chemistry Development, Inc., ACD/PhysChem Suite software, version 12.01.
[e] *In vitro* recovery for the type of probe, not for the matrix itself.
[f] Apparent value calculated from group data.

Fig. 3. The comparison between the cell models. The permeability category limits (High, Med, Low) were set according to Polli et al. (2000) in the filter insert systems (Caco-2 and MDCKII-MDR1 models), and according to Eddy et al. (1997) in the side-by-side diffusion system (BBMEC).

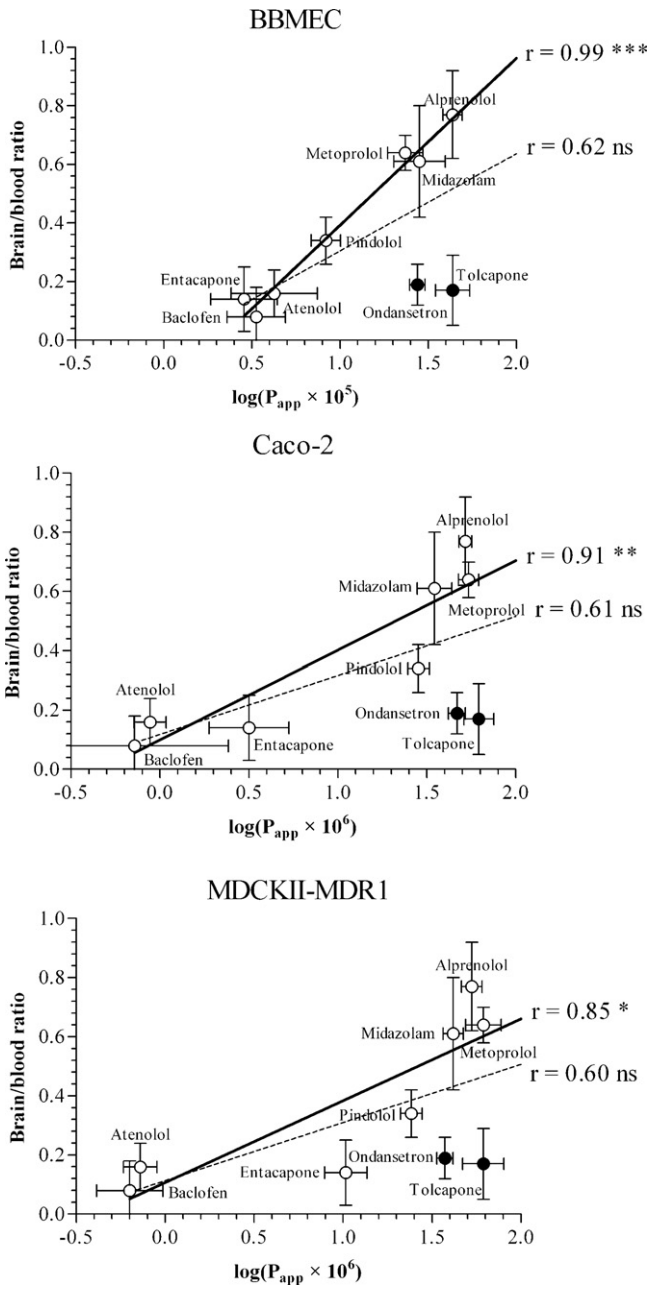

Fig. 4. *In vivo* relevance of BBMEC, Caco-2 and MDCKII-MDR1 cell models (mean ± S.D.). Brain/blood ratios *in vivo* are presented as a function of log-transformed P_{app}. The data points excluded from the linear regression and correlation analyses are marked with black symbols (●). The solid line (—) represents the linear regression after the exclusion of the outliers, and the dotted line (- - -) represents the linear regression with all data points included (r = Pearson correlation coefficient: $^{***}P < 0.0001$; $^{**}P < 0.01$; $^{*}P < 0.05$, ns = not significant).

Table 4
The rank order of the model drugs from the highest (Rank #1) to the lowest (Rank #9) permeability in each model.

Rank #	BBMEC	Caco-2	MDCKII-MDR1	Microdialysis
1	Tolcapone	Tolcapone	Tolcapone	Alprenolol
2	Alprenolol	Metoprolol	Metoprolol	Metoprolol
3	Midazolam	Alprenolol	Alprenolol	Midazolam
4	Ondansetron	Ondansetron	Midazolam	Pindolol
5	Metoprolol	Midazolam	Ondansetron	Ondansetron
6	Pindolol	Pindolol	Pindolol	Tolcapone
7	Atenolol	Entacapone	Entacapone	Atenolol
8	Baclofen	Atenolol	Atenolol	Entacapone
9	Entacapone	Baclofen	Baclofen	Baclofen

compounds were the same with the exception of the P-gp substrate ondansetron, which was classified as a medium permeability compound in the MDCKII-MDR1 model, and pindolol, which was categorized into the medium range in both models. Atenolol, baclofen, entacapone and the low permeability reference compound sucrose were ranked as low permeability compounds in the Caco-2 and MDCKII-MDR1 models whereas in the BBMEC model, none of the drugs studied reached the low permeability category. This clearly demonstrates that the BBMEC model is leakier than the Caco-2 and MDCKII-MDR1 models. Taken together, the rank order of the model drugs is similar in all three *in vitro* cell models despite the fact that the permeability ranges between models are clearly different under the present experimental conditions.

The P_{app} values are mostly influenced by the physicochemical characteristics of the drugs and by the permeation characteristics of the cells *per se* but partly also by the experimental set-up. The permeation experiments were performed using procedures typical for each cell model, thus resulting in different experimental set-ups (*i.e.,* BBMEC in side-by-side diffusion chambers (Hansen et al., 2002) and Caco-2 or MDCKII-MDR1 in filter insert system (Braun et al., 2000)). The side-by-side diffusion chamber system is composed of symmetrical donor and receiver chambers both of which are mechanically stirred whereas the filter insert system with asymmetrical upper donor and lower receiver chambers is agitated on an external shaking platform. Differences in stirring action and geometry of the permeation systems will lead to different drug diffusion characteristics and differences in the unstirred water layer (UWL) between the *in vitro* models have been found to contribute to the determined permeability coefficients (Korjamo et al., 2008). This implicates that the limited stirring action and higher UWL in the filter insert system, the high permeability values which are obtained in the side-by-side system, cannot be achieved, which is in agreement with our findings. A thinner UWL, as in the BBMEC model, may more closely resemble that of the *in vivo* situation, where the thickness of the UWL has been suggested to be as low as < 1 μm (Avdeef et al., 2004). Thus, the more efficient stirring in the BBMEC model compared to those in Caco-2 and MDCKII-MDR1 models may partly explain its apparently better *in vitro–in vivo* correlation coefficient.

To determine the *in vivo* rank order of the model drugs in the present study, a dual probe microdialysis method was used. To monitor the unbound drug concentrations on both sides of the blood–brain barrier simultaneously as a function of time, one probe was inserted into the rat brain and another one into a peripheral blood vessel. The *in vivo* unbound brain/blood ratios of the nine model drugs varied from 0.08 (baclofen) to 0.77 (alprenolol). The results indicate that the present straightforward pharmacokinetic microdialysis approach is able to discriminate drugs with different abilities to cross the blood–brain barrier and to enter into the brain. The major advantage of the pharmacokinetic microdialysis method as an *in vivo* measure is that the resulting unbound fraction is comparable to the *in vitro* situation in which the drug concentrations also represent unbound concentrations.

The *in vivo* relevance of the *in vitro* P_{app} values was assessed by correlating the log-normalized P_{app} values determined in the three cell models vs. the unbound brain/blood ratios determined by *in vivo* microdialysis. The best correlation between the log-normalized *in vitro* P_{app} values and the unbound brain/blood ratios was observed in BBMEC model (r = 0.99), followed by Caco-2 (r = 0.91) and MDCKII-MDR1 (r = 0.85) models when ondansetron and tolcapone were excluded from the analysis. However, the differences between the correlation coefficients cannot be considered as significant; it is more likely to result from the distribution of the rather small amount of data points in the regression analysis. None of the correlation coefficients were statistically significant in the presence of ondansetron and tolcapone. These two drugs were found to have high permeabilities in all three cell models, but

very low brain/blood ratios *in vivo*. Thus, their *in vitro–in vivo* correlation was poor. The low unbound brain/blood ratios values of ondansetron (a substrate for P-gp (Schinkel et al., 1996)) and tolcapone (own preliminary data on efflux protein interactions, data not shown) *in vivo* can be explained by the function of the efflux transporters such as the P-gp. Efflux transporters restrict the transport of their substrates from blood circulation into the brain, thus lowering the $AUC_{brainECF\ 0-\infty}$ values determined with *in vivo* microdialysis method. As a result, the AUC_{brain}/AUC_{blood} ratios decline.

The drug concentrations attributed to the *in vivo* model are physiologically relevant, and normal function of active transport processes can be assumed. In the *in vitro* models, in contrast, it is likely that the active processes are saturated due to the rather high donor concentrations used in our experiments, or, alternatively, certain active efflux processes may exist but the passive permeability exceeds significantly the active efflux processes (Eytan et al., 1997; Lentz et al., 2000). Taken together, these results suggest that especially when relatively high concentrations are used *in vitro*, the present cell models may be unable to quantify active transport and they mainly rank drugs on the basis of their passive permeabilities. This also emphasizes the importance of *in vivo* methods in assessing the ability of drugs to cross the blood–brain barrier: although *in vitro* methods are advantageous during early drug discovery, *in vivo* methods clearly provide more extensive and diverse information of the new drug candidates (Reichel, 2006).

In conclusion, the present study demonstrates the *in vivo* relevance of the simple unidirectional P_{app} parameter for the nine model drugs studied. There is a clear correlation between the P_{app} values determined in each *in vitro* model and the unbound brain/blood ratios determined by *in vivo* microdialysis. Despite the substantial differences in the absolute *in vitro* P_{app} value ranges and irrespective of the method used (side-by-side vs. filter insert system), the capability of the cell models to rank order drugs is similar. Thus, the additional value offered by true endothelial cell model (BBMEC) remains obscure. The present results also highlight the need for both *in vitro* as well as *in vivo* methods in the characterization of blood–brain barrier passage of novel drug candidates.

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