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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