The Grasshopper: A Novel Model for Assessing Vertebrate Brain Uptake

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Received April 10, 2013; accepted May 10, 2013

ABSTRACT

The aim of the present study was to develop a blood-brain barrier (BBB) permeability model that is applicable in the drug discovery phase. The BBB ensures proper neural function, but it restricts many drugs from entering the brain, and this complicates the development of new drugs against central nervous system diseases. Many in vitro models have been developed to predict BBB permeability, but the permeability characteristics of the human BBB are notoriously complex and hard to predict. Consequently, one single suitable BBB permeability screening model, which is generally applicable in the early drug discovery phase, does not yet exist. A new refined ex vivo insect-based BBB screening model that uses an intact, viable whole brain under controlled in vitro-like exposure conditions is presented.

This model uses intact brains from desert locusts, which are placed in a well containing the compound solubilized in an insect buffer. After a limited time, the brain is removed and the compound concentration in the brain is measured by conventional liquid chromatography-mass spectrometry. The data presented here include 25 known drugs, and the data show that the ex vivo insect model can be used to measure the brain uptake over the hemolymph-brain barrier of drugs and that the brain uptake shows linear correlation with in situ perfusion data obtained in vertebrates. Moreover, this study shows that the insect ex vivo model is able to identify P-glycoprotein (Pgp) substrates, and the model allows differentiation between low-permeability compounds and compounds that are Pgp substrates.

Introduction

The vertebrate blood brain barrier (BBB) is composed of capillary endothelial cells that control the entry of nutrients and xenobiotics to the brain, and it ensures an optimal environment for proper neural function (Abbott et al., 2006, 2010). However, the BBB creates a great obstacle for the medical treatment of diseases related to the central nervous system (CNS) and is recognized as a major obstacle in the discovery of new drugs against CNS-related diseases (Geldenhuys et al., 2012). Therefore, a number of cell based in vitro models have been developed and used as tools in the drug-discovery screening process (Polli et al., 2001; Weiss et al., 2003; Mensch et al., 2009). Two commonly used systems are the renal cell lines LLC-PK1 and MDCK. Both are easy to grow, and this makes them attractive for industrial use. However, these cells are of epithelial origin, and compared with barrier endothelial cells, epithelial cells display differences in morphology, tight junction organization, and transporter expression (Garberg et al., 2005; Cecchelli et al., 2007; Abbott et al., 2008; Liu et al., 2008). Recently, we presented an insect-based BBB screening model that uses an intact whole brain under controlled in vitro–like exposure conditions (Nielsen et al., 2011). As in vertebrates, the protection of the insect CNS requires a tight brain barrier containing influx and efflux transporter proteins, which control elements entering the brain (Banerjee and Bhat, 2007; Bundgaard and Abbott, 2008; Stork et al., 2008; DeSalvo et al., 2011). The insect brain barrier consists of glia cells, which are the most abundant cell type in the vertebrate CNS. Paracellular diffusion in insects is controlled by septate/tight junctions that are homologous to tight junctions controlling paracellular diffusion in vertebrates (Wu and Beitel, 2004; Daneman and Barres, 2005; Freeman and Doherty, 2006).

Transporters are present in the insects, and it has been shown that the human MDR1 antibody (c219) binds to the MDR65 (i.e., an insect MDR1 homolog present in the insect brain barrier) (Mayer et al., 2009). Functionally, we have shown that the Pgp inhibitor verapamil blocks the efflux of Pgp substrates (Nielsen et al., 2011). This study concluded that insect models can be used as models to identify Pgp substrates. The structural and functional similarities of the vertebrate and invertebrate BBB enable the use of invertebrates as advanced screening models for BBB permeability determinations.

This work was supported by the Danish National Advanced Technology Foundation [Grant 023-2011-3].

dx.doi.org/10.1124/jpet.113.205476.

ABBREVIATIONS: BBB, blood-brain barrier; CNS, central nervous system; Ctot, total brain concentration; KO, knockout; LC-MS, liquid chromatography-mass spectrometry; MDCK, Madin-Darby canine kidney; MDCK-MDR1, MDR1-transfected MDCK; MDR1, multidrug resistance protein-1; $P_{app}$, apparent permeability coefficient; PS, permeability-surface area; Pgp, P-glycoprotein.
Previously, two different insect-based BBB permeability models have been reported (Nielsen et al., 2011). One model is a traditional in vivo model where the substance of interest is injected directly into the abdomen, and the brain and the body fluid (hemolymph) concentrations are measured. The brain-hemolymph ratio gives an estimate of the ability of the compound to permeate the BBB. However, as in vertebrates, the insect in vivo results are influenced by a number of parameters, like unspecific protein binding, metabolism, distribution, and elimination, making it complex to translate brain-hemolymph ratios into BBB permeability. To reduce this complexity, we have developed a second model, the ex vivo model. In this model, the intact brain is dissected from the insect and placed in a well containing the compound of interest (Fig. 1). Insects are characterized by their exoskeleton, where the organs are surrounded by the hemolymph, which is circulated by the heart. No capillaries are present in insects, and they do not depend on circulation of the hemolymph through the organs, allowing dissection of the brain before compound exposure, and the absence of capillaries in the insect brain eliminates any contribution from residual blood in the brain.

The ex vivo BBB permeability model is unique because of the lack of biologic events outside the brain. Despite this, the brain barrier is intact, and all mechanisms and biologic events inside the brain are preserved.

In this study, we use the ex vivo grasshopper model to measure brain uptake to assess the BBB permeability of 25 known drugs. Insect brain uptake is compared with perfusion data from the literature, and a linear correlation between ex vivo and in situ brain uptake is found. The ex vivo conditions ensure that the exposure concentration is controllable and constant during an experiment, and this feature is used to investigate concentration-dependent permeabilities. The ex vivo data at high exposure concentrations correlate remarkably well to vertebrate in situ data. Moreover, it is shown that ex vivo data obtained at low exposure concentrations allow identification of Pgp substrates by coadministration with the Pgp inhibitor verapamil.

Materials and Methods

Animals. Desert locusts, Schistocerca gregaria (L), were obtained from a commercial animal breeder (Petra Aqua, Prague, Czech Republic). At arrival, the locusts were housed under crowded conditions in an insect room and adapted to a 12:12 hours dark/light cycle. The locusts were maintained in colonies at a local terrarium temperature of 30–34°C. The animals were fed Chinese cabbage, fresh grass, and wheat bran ad libitum. All experiments were carried out on fifth instar locusts 2 to 3 weeks after adult emergence.

Chemicals. All drugs used in the insect ex vivo studies and HEPES were purchased from Sigma-Aldrich (Stockholm, Sweden). The drugs used in MDCK-MDR1 experiments to determine were available from Lundbeck A/S in-house sources. All other chemicals were analytical reagent grade.

Reference Set Selection. Twenty-five known drugs were selected from the literature with the objective of having a balanced number of known CNS and non-CNS drugs. To avoid any ambiguity, the therapeutic indication of the selected compounds was either CNS or peripheral. The selected drugs are representative for the marketed drugs in terms of LogP, molecular weight, and number of H-bond donors and acceptors, and the set contains neutral, acidic, and basic drugs. Moreover, the selected set contains known CNS drugs, which are known efflux substrates and passively diffusing compounds.

MDCK-MDR1 Experiments. MDR1-MDCK cells originate from the transfection of Madin-Darby canine kidney (MDCK) cells with the MDR1 gene, the gene encoding for Pgp. MDCK-MDR1 cells (obtained from Piet Borst at the Netherlands Cancer Institute, Amsterdam, The Netherlands) were seeded onto polyethylene membranes in 96-well BD insert systems at 2×10⁶ cells/cm² for 4–6 days for confluent cell monolayer formation. Test compounds (2 μM) were applied to the top (A) or bottom (B) side of the cell monolayer. Permeation of the test compounds from A to B direction or B to A direction was determined in triplicate over a 150-minute incubation at 37°C and 5% CO₂ with a relative humidity of 95%. In addition, the efflux ratio of each compound was determined. Test and reference compounds were quantified by LC-MS or LC-MS/MS analysis based on the peak area ratio of analyte/internal standard.

Ex Vivo Experiments.

Test solution preparation. Stock solutions were prepared by dissolving the test compounds in dimethylsulfoxide. Final test solution concentrations were obtained by diluting stock solutions with insect buffer [147 mM NaCl, 10 mM KCl, 4 mM CaCl₂, 3 mM NaOH, and 10 mM HEPES, pH 7.2].

Ex vivo experiment procedure. The brain was dissected by cutting off the frontal part of the head through the esophagus. The three merged ganglia of the brain (protocerebrum, deutocerebrum, and tritocerebrum) were prepared by using fine forceps. The brain was dissected free from fat and placed in a Microwell plate (96U Microwell; Nunc Microwell Plates; Sigma-Aldrich) containing 250 μL of the test compound dissolved in the insect buffer. The experiments are run at 30°C in a block thermostat (HLC Technologies, Pforzheim, Germany). After a standard exposure time of 15 minutes, the brains were removed from the wells and washed twice in ice-cold insect buffer. Before transfer to the test tubes, the neural lamella surrounding the brain was removed. After the addition of 150 μL of 2% zinc sulfate in 50% methanol, the brain tissue was homogenized by means of ultrasonication (Bandelin Electronic, Berlin, Germany) for 8 seconds at a power of 19%. After centrifugation at 10,000 rpm for 5 minutes at 4°C, the supernatant was analyzed by high-performance LC-MS. The ex vivo studies are performed as triple tests with two brains pooled in each test tube (i.e., a total of six brains in each study) (Fig. 1).

Analysis of the Quantity of Drugs in Insect Brain Tissue.

The supernatants from the brain homogenates were analyzed by LC-MS using an Agilent 1200 HPLC coupled to an MSD 1100 detector (Agilent Technologies, Walbronn, Germany). The chromatographic column was a Phenomenex Kinex C₁₈ (2.6-μm particles with 2.6-μm particles, and was kept at 30°C. The mobile phase A was 0.1% formic acid, and the mobile phase B was 0.1% formic acid in methanol; analysis was performed using gradient elution from 5% to 90% mobile phase B. Detection was performed in single ion monitoring mode using the appropriate [M+1] ions in positive mode.

Calculation of Brain Compound Concentration. The concentration measured by LC-MS equals the brain concentration of two brains diluted in 150 μL of 50% MeOH with 2% v/v ZnSO₄ and 500 nM caffeine. The weight of an average desert locust brain is 1.6 mg, and it is assumed that this weight equals a volume of 1.6 ml. Thus, to calculate the compound brain concentration, the concentration measured by LC-MS is multiplied by 150 μL and divided by 2 x 1.6 μL.

Statistical Analysis. All values are expressed as mean ± S.D. To determine statistical significantly differences among the experimental groups, the single-tailed student’s t test was used. A P value of less than 0.05 was termed significant.

Results

Table 1 details the compound reference set used in the present study. The set includes 13 known CNS drugs and 12 known peripheral drugs, and they cover a wide range of physicochemical properties. The reference set includes acidic, neutral, basic, and zwitterionic substances. The molecular
mass varies from 194 to 1203 g/mol, and the lipophilicity varies from 2.2 to 5.5.

**Results from MDCK-MDR1.** The apparent permeability coefficients (P_{app}) were obtained by MDCK-MDR1 cells for 14 compounds using the method described already, and the P_{app} values for seven were extracted from the literature (Di et al., 2009) (Table 1). The measured permeability coefficients range from 78 to \(10^{-6}\) cm/s. Eight of the known CNS drugs, including warfarin, are characterized by a P_{app} greater than \(10^{-6}\) cm/s. The P_{app} values for four CNS drugs were as follows: risperidone, fluoxetine, amitriptyline, and desipramine are in the range between 5 and \(10^{-6}\) cm/s. Compounds with P_{app} lower than \(10^{-6}\) cm/s are paroxetine and eight peripheral acting drugs.

**Results from Ex Vivo Brain Uptake.** Uptake in the insect brain was measured after 15 minutes in a 10 \(\mu\)M exposure concentration of the compound test solution. Compounds characterized by high concentrations in the insect brain are assumed to have high BBB permeability, and they are likely to enter the vertebrate brain. The brain concentrations

### Table 1

<table>
<thead>
<tr>
<th>Drug (CAS No.)</th>
<th>Class</th>
<th>MW</th>
<th>cLogP*</th>
<th>Ctot 3(\mu)M (S.D.)</th>
<th>Ctot 3(\mu)M + Verapamil (S.D.)</th>
<th>Ctot 10(\mu)M (S.D.)</th>
<th>P_{app} (10^{-6}) MDCK-MDR1</th>
<th>LogPS In Situ(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil (S.D.)</td>
<td>Neutral</td>
<td>781</td>
<td>1.4</td>
<td>0.2 (0.0)</td>
<td>0.8 (0.1)</td>
<td>0.1(^b)</td>
<td>12(^b)</td>
<td>-4.5</td>
</tr>
<tr>
<td>Cimetidine (51481-61-9)</td>
<td>Neutral</td>
<td>252</td>
<td>0.2</td>
<td>0.2 (0.0)</td>
<td>0.4 (0.0)</td>
<td>0.1(^b)</td>
<td>12(^b)</td>
<td>-4.1</td>
</tr>
<tr>
<td>Cetirizine (53179-11-6)</td>
<td>Basic</td>
<td>787</td>
<td>0.6</td>
<td>2.3 (0.8)</td>
<td>6.2 (0.6)</td>
<td>2(^b)</td>
<td>12(^b)</td>
<td>-1.7</td>
</tr>
<tr>
<td>Quinidine (56-54-2)</td>
<td>Basic</td>
<td>324</td>
<td>2.8</td>
<td>1.9 (0.2)</td>
<td>3.2 (0.8)</td>
<td>12(^b)</td>
<td>12(^b)</td>
<td>-2.1</td>
</tr>
<tr>
<td>Warfarin (81-81-2)</td>
<td>Neutral</td>
<td>308</td>
<td>2.9</td>
<td>1.6 (0.1)</td>
<td>4.7 (1.2)</td>
<td>78(^e)</td>
<td>12(^b)</td>
<td>12(^b)</td>
</tr>
<tr>
<td>Citalopram (59729-33-8)</td>
<td>Basic</td>
<td>324</td>
<td>3.1</td>
<td>1.1 (0.1)</td>
<td>4.6 (0.1)</td>
<td>11(^f)</td>
<td>12(^b)</td>
<td>12(^b)</td>
</tr>
<tr>
<td>Risperidone (106266-06-2)</td>
<td>Basic</td>
<td>410</td>
<td>2.7</td>
<td>4.3 (0.3)</td>
<td>8.2 (0.5)</td>
<td>5.0(^b)</td>
<td>12(^b)</td>
<td>-1.8</td>
</tr>
<tr>
<td>Loperamide (53179-11-6)</td>
<td>Basic</td>
<td>787</td>
<td>0.6</td>
<td>2.3 (0.8)</td>
<td>6.2 (0.6)</td>
<td>2(^b)</td>
<td>12(^b)</td>
<td>-1.7</td>
</tr>
<tr>
<td>Quinidine (56-54-2)</td>
<td>Basic</td>
<td>324</td>
<td>2.8</td>
<td>1.9 (0.2)</td>
<td>3.2 (0.8)</td>
<td>12(^b)</td>
<td>12(^b)</td>
<td>-2.1</td>
</tr>
<tr>
<td>Warfarin (81-81-2)</td>
<td>Neutral</td>
<td>308</td>
<td>2.9</td>
<td>1.6 (0.1)</td>
<td>4.7 (1.2)</td>
<td>78(^e)</td>
<td>12(^b)</td>
<td>12(^b)</td>
</tr>
<tr>
<td>Citalopram (59729-33-8)</td>
<td>Basic</td>
<td>324</td>
<td>3.1</td>
<td>1.1 (0.1)</td>
<td>4.6 (0.1)</td>
<td>11(^f)</td>
<td>12(^b)</td>
<td>12(^b)</td>
</tr>
<tr>
<td>Supravision (34841-39-9)</td>
<td>Basic</td>
<td>240</td>
<td>3.2</td>
<td>1.8 (0.4)</td>
<td>7.9 (2.2)</td>
<td>29(^f)</td>
<td>12(^b)</td>
<td>-1.5</td>
</tr>
<tr>
<td>Trizodone (19794-93-5)</td>
<td>Basic</td>
<td>372</td>
<td>3.9</td>
<td>4.9 (1.8)</td>
<td>9.2 (1.8)</td>
<td>17(^b)</td>
<td>12(^b)</td>
<td>-1.5</td>
</tr>
<tr>
<td>Haloperidol (52-86-8)</td>
<td>Basic</td>
<td>376</td>
<td>3.8</td>
<td>3.4 (1.0)</td>
<td>13.4 (0.3)</td>
<td>28(^d)</td>
<td>12(^b)</td>
<td>-1.5</td>
</tr>
<tr>
<td>Carbamazepine (298-46-6)</td>
<td>Neutral</td>
<td>236</td>
<td>2.4</td>
<td>5.9 (0.3)</td>
<td>16.0 (0.9)</td>
<td>31(^b)</td>
<td>12(^b)</td>
<td>-1.3</td>
</tr>
<tr>
<td>Propranolol (525-66-6)</td>
<td>Basic</td>
<td>259</td>
<td>2.8</td>
<td>1.1 (0.2)</td>
<td>8.1 (0.8)</td>
<td>28(^b)</td>
<td>12(^b)</td>
<td>-2.0</td>
</tr>
<tr>
<td>Fluoxetine (54910-89-3)</td>
<td>Basic</td>
<td>309</td>
<td>4.6</td>
<td>5.9 (2.2)</td>
<td>13.8 (0.8)</td>
<td>18(^b)</td>
<td>12(^b)</td>
<td>-1.2</td>
</tr>
<tr>
<td>Amitriptyline (50-48-6)</td>
<td>Neutral</td>
<td>277</td>
<td>4.9</td>
<td>5.9 (2.2)</td>
<td>13.8 (0.8)</td>
<td>18(^b)</td>
<td>12(^b)</td>
<td>-1.2</td>
</tr>
<tr>
<td>Atanol (29122-68-7)</td>
<td>Basic</td>
<td>266</td>
<td>-0.1</td>
<td>0.2 (0.0)</td>
<td>0.1(^b)</td>
<td>12(^b)</td>
<td>12(^b)</td>
<td>12(^b)</td>
</tr>
<tr>
<td>Paroxetine (61869-08-7)</td>
<td>Basic</td>
<td>329</td>
<td>4.2</td>
<td>6.5 (2.1)</td>
<td>25.0 (1.4)</td>
<td>5.0(^b)</td>
<td>12(^b)</td>
<td>-1.5</td>
</tr>
<tr>
<td>Ranitidine (68357-35-5)</td>
<td>Basic</td>
<td>314</td>
<td>0.7</td>
<td>0.2 (0.0)</td>
<td>0.3(^b)</td>
<td>12(^b)</td>
<td>12(^b)</td>
<td>-1.3</td>
</tr>
<tr>
<td>Caffeine (58-08-2)</td>
<td>Neutral</td>
<td>194</td>
<td>0.0</td>
<td>1.7 (0.1)</td>
<td>1.9 (0.2)</td>
<td>20(^d)</td>
<td>12(^b)</td>
<td>12(^b)</td>
</tr>
<tr>
<td>Desipramine (50-47-5)</td>
<td>Basic</td>
<td>266</td>
<td>4.5</td>
<td>1.8 (0.4)</td>
<td>10.0 (2.8)</td>
<td>5.0(^b)</td>
<td>12(^b)</td>
<td>12(^b)</td>
</tr>
<tr>
<td>Lincomycin (154-21-2)</td>
<td>Basic</td>
<td>407</td>
<td>1.3</td>
<td>0.3 (0.1)</td>
<td>0.3(^b)</td>
<td>12(^b)</td>
<td>12(^b)</td>
<td>12(^b)</td>
</tr>
<tr>
<td>Norfloxacin (70458-96-7)</td>
<td>Zwitterion</td>
<td>319</td>
<td>-0.8</td>
<td>0.2 (0.0)</td>
<td>0.3 (0.3)</td>
<td>0.1(^b)</td>
<td>12(^b)</td>
<td>12(^b)</td>
</tr>
<tr>
<td>Methotrexate (59-05-2)</td>
<td>Acid</td>
<td>454</td>
<td>-0.5</td>
<td>0.2 (0.0)</td>
<td>0.2 (0.0)</td>
<td>0.1(^b)</td>
<td>12(^b)</td>
<td>12(^b)</td>
</tr>
<tr>
<td>Dexamethasone (50-02-2)</td>
<td>Neutral</td>
<td>392</td>
<td>1.8</td>
<td>0.2 (0.0)</td>
<td>1.0 (0.2)</td>
<td>2.6(^b)</td>
<td>12(^b)</td>
<td>12(^b)</td>
</tr>
<tr>
<td>Cyclosporin A (59865-13-3)</td>
<td>Neutral</td>
<td>1203</td>
<td>&gt;4</td>
<td>0.4 (0.0)</td>
<td>2.6 (0.3)</td>
<td>12(^b)</td>
<td>12(^b)</td>
<td>12(^b)</td>
</tr>
</tbody>
</table>

CAS, Chemical Abstracts Service; MW, molecular weight.

* cLogP (calculated lipophilicity) was calculated using the MOE (Molecular Operating System, 2012).

** Present work.

\(^d\) Di et al., 2009.

\(^f\) Dagenais et al., 2009.
are displayed in Fig. 2; the data showed that all CNS active compounds in the reference set are present in the insect brain in high concentrations. Generally, the peripheral-acting compounds in the reference set were present in the insect brain in low concentrations (i.e., reflecting low brain uptake in vertebrates). From the data shown in Fig. 2, it is seen that there is a group of compounds that may be classified as intermediate permeating compounds. The intermediate group includes the peripheral drugs cetirizine, quinidine, and loperamide. These three drugs are all known Pgp substrates in vertebrates. Previous studies showed that high exposure concentrations in the ex vivo model may saturate transporters, and this may diminish the efflux or influx mechanism (Nielsen et al., 2011).

To reduce the risk of saturation of the transporters, we decided to study the brain uptake at lower exposure concentrations.

In Fig. 3, the measured insect brain concentrations of the reference compounds are shown when using exposure concentrations of 3 μM. A separation is seen between peripheral and CNS active drugs. All peripheral compounds are those present in lowest concentrations in the insect brain.

As expected, the measured insect brain concentrations are highly dependent on the exposure concentration, and for all compounds in the reference set, the measured insect brain concentration at 3 μM exposure is lower than at 10 μM. Reduction of the exposure concentration from 10 to 3 μM does not always lead to a 3-fold reduction of the brain concentration.
Identification of Pgp Substrates: To identify the Pgp substrates in the present reference set, the compounds were coadministered with the Pgp inhibitor verapamil, which blocks the efflux of Pgp substrates. In this study, the brain uptake of compounds that are not Pgp substrates should not be influenced by coadministration with verapamil, whereas an increased brain concentration is expected for Pgp substrates. Here we used the compound exposure concentration of 3 μM and verapamil concentration of 25 μM. To identify Pgp substrates, we calculated the ratio between the measured brain concentrations, i.e. the total concentration (Ctot), when Pgp substrates are removed from the intermediate group. At 10 μM, the intermediate group includes both CNS drugs and Pgp substrates. Previous studies have shown that high exposure concentration of Pgp substrates may saturate the transporter, which diminishes the Pgp efflux (Nielsen et al., 2011). Thus, at 10 μM, Pgp substrates may start to saturate of the efflux mechanism, and this potentially leads to the characterization of Pgp substrates as having intermediate permeability. Reduction of the exposure concentration reduces the risk for saturation, and at 3 μM, the Pgp substrates are removed from the intermediate group. At 3 μM, all peripheral-acting drugs are characterized as those displaying the lowest brain uptake.

BBB Permeability: The exposure time in the insect ex vivo model is 15 minutes, and the exposure concentration is considered constant during the entire experiment. In addition, the model does not contain any plasma proteins, and no metabolic enzymes are present in the exposure solution. Thus, as with in vitro permeability models, the ex vivo model is expected to correlate with permeability-surface area (PS) obtained by in situ perfusion experiments. PS is the product of BBB permeability and the microcapillary-specific surface area, and it is the gold standard for permeability measurements (Dagenais et al., 2009; Abbott et al., 2012). To evaluate the capability of the ex vivo model to predict BBB permeabilities, we used rodent perfusion in situ data extracted from Dagnais et al. (2009). It was previously shown that there is a close 1:1 relationship between LogPS obtained in mice and rats (Murakami et al., 2000). Thus, despite the in situ data used in this study being generated at different laboratories and coming from different species, we still consider them useful as benchmarking data. We have used in situ data, when it is available, obtained in either MDR1 knockout (KO) mice or from experiments where Pgp has been blocked by a Pgp inhibitor (Table 1).

In vitro Papp values have been obtained by using MDCK-MDR1 cells characterized by overexpression of Pgp. Thus, correlation between Papp and in situ LogPS is expected to be seen only for compounds known to be non-Pgp substrates. However, as seen in Fig. 4A, no correlation is found between the in situ data extracted from Dagenais et al. (2009) and the MDCK-MDR1 data extracted from the work of Di et al. (2009) or generated in this study. As shown by Di et al., the MDCK-MDR1 data do not correlate with in situ perfusion data, and a number of compounds are detected as false positives and even more problematic false negatives (i.e., CNS drugs that are low or nonpermeating in the MDCK-MDR1 assay).

The insect ex vivo brain uptake data at 10 μM show a strong correlation (R² = 0.88) with the in situ data (Fig. 4B). These
data indicate that the ex vivo insect model gives a quantitative measurement of the brain uptake that corresponds to in situ data. At 10 μM exposure concentration, the Pgp transporters start to be saturated. Thus, the observed remarkable good correlation between the in situ LogPS and Ctot at 10 μM is likely due to the fact that the Pgp efflux is diminished in both cases.

It is well known that transporter systems may be saturated at high exposure concentrations, and this will influence BBB permeability measurements (Nielsen et al., 2011, Mahar Doan et al., 2002). To avoid saturation of the transporters in the ex vivo system, the exposure concentration was reduced to 3 μM, and BBB permeability plus Pgp efflux was investigated. As seen in Fig. 4C, the correlation coefficient between Ctot at 3 μM and LogPS is lower than at 10 μM exposure (i.e., $R^2 = 0.83$ vs. $R^2 = 0.88$, respectively). Coexposure at 3 μM with verapamil increases the correlation between the measured insect brain concentration and LogPS, $R^2 = 0.85$. Thus, there is a slightly stronger correlation between LogPS and insect brain concentrations when the insect transporters are partly saturated (at 10 μM) or when the insect Pgp analog is blocked by coadministration with verapamil.

In MDCK, permeability is assessed by a cell layer, whereas the ex vivo and in situ models use brains containing brain tissues. The brain uptake measured in the insect ex vivo is influenced by the brain tissue binding (unpublished results), and the correlation to in situ data makes it reasonable to believe that brain tissue binding also has an impact on the in situ perfusion data. Thus, the MDCK and the ex vivo models may complement each other where the former gives a raw permeability assessment and the latter an alternative to in situ or in vivo brain-plasma ratio measurements.

Brain tissue has a high content of fat, which may increase the uptake of lipophilic compounds in the brain. As seen in Fig. 5, there is a linear dependency between lipophilicity and uptake measured in situ and ex vivo at 10 μM; correlation coefficients are 0.70 and 0.64, respectively. Ex vivo data obtained at 3 μM exposure hardly correlate to lipophilicity ($R^2 = 0.46$), and the correlation coefficient increases to 0.57 using 3 μM exposure plus verapamil. This finding suggests that lipophilicity is the dominant factor determining the BBB permeability when the transporter systems are blocked either when using MDR1 KO mice, by saturation, or by blocking the Pgp system. At 3 μM, the transporters in the insect brain are active and can be blocked by coadministration with verapamil, which increases the insect brain concentration for known Pgp substrates (Table 2). As seen in Table 2, four known CNS active compounds—trazodone, citalopram, risperidone, and desipramine—are found to be Pgp substrates. However, it is obvious from the data obtained in the insect model that despite these compounds being Pgp substrates, they are still present in significant amounts in the insect brain. Thus, the ex vivo data suggest that these drugs are likely to have an effect on the CNS.

The insect brain consists of glia cells. This, together with the species differences, implies that there may be differences between the functionality of the Pgp in rodents and insects. However, apart from trazodone, the insect data are in accordance with the classification of the compounds obtained from Pgp-KO mice or when using the B/A/A-B ratio from MDCK-MDR1 (Mahar Doan et al., 2002; Doran et al., 2005; Feng et al., 2008). These data suggest that the insect ex vivo model can be used to identify Pgp substrates. However, further investigations of the sequence homology between the

![Fig. 4. Correlations between LogPS extracted from Dagenais et al. (2009) and the different permeability models used in this work: apparent permeability measured using MDCK-MDR1 cells (A), logarithm of grasshopper brain concentration obtained after 10 μM exposure concentration (B), logarithm of grasshopper brain concentration obtained after 3 μM exposure concentration (C), logarithm of grasshopper brain concentration + verapamil (D). Only compounds known not to be Pgp substrates are included in (B).](image-url)
grasshopper and vertebrate Pgps are needed to provide a deeper understanding of the Pgp similarities.

**How to Use the Ex Vivo Model.** The data obtained with the ex vivo insect model emphasizes the complexity of the transport mechanisms. As in vertebrates, grasshopper brain uptake shows that Pgp substrates can be CNS drugs (DeSalvo et al., 2011). On the other hand, Pgp substrates can saturate the Pgp transporters, and these may falsely be considered as potential CNS drugs. Substrates have different affinities to the different transporter systems, and the concentration at which the transporters are saturated is compound dependent (Matsson et al., 2009). The data obtained here suggest that the insect ex vivo model may be useful to identify and test the saturation of transport mechanisms.

The insect ex vivo brain uptake model has a higher degree of correlation with in situ perfusion data than does MDCK-MDR1. A main difference between the insect ex vivo and MDCK models is the cell layer, which in the insect brain barrier consists of glia cells, whereas MDCK is based on epithelial cells. In the ex vivo model, the biologic events inside the brain are still active. These events include nonspecific binding to brain lipids and proteins, and the brain uptake results are influenced by nonspecific binding to the brain tissue (i.e., higher brain tissue binding will push the equilibrium over the BBB toward a higher brain concentration). However, the insect brain uptake does not reveal anything about the free fraction in the brain. To obtain knowledge about the potential therapeutic effects of a compound, both the insect uptake as well as the brain free fraction should be determined.

In conclusion, the developed insect ex vivo BBB model allows classification of compounds as high, low, or intermediate permeating compounds. At high exposure concentrations, the transport efflux may be diminished and the ex vivo data correlates to in situ data obtained in rodents. Thus, at 10 \( \mu \text{M} \), the ex vivo model is useful for BBB permeability measurements. At 3 \( \mu \text{M} \), the Pgp efflux mechanism is pronounced, and there is a lower correlation to in situ data. Pgp substrates can be identified by coadministration with a Pgp inhibitor like verapamil.

**Acknowledgments**

The authors thank Kirsten Andersen, chief technician, and Marianne Reni Andersen, technician, University of Copenhagen, for assisting the LC-MS analysis and Laurent David, principal scientist, Lundbeck A/S, for computational support.

**References**


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