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ABSTRACT

The blood-brain barrier (BBB) permeabilities of 11 compounds were measured both in vitro with a newly developed coculture-based model of human BBB and in vivo with positron emission tomography (PET). The 11 compounds were fluoropyridinyl derivatives labeled with the positron-emitter fluorine-18, \(^{18}\text{F}\)-A-85380 \text{[}2-^{18}\text{F}]\text{fluoro-3-[2(S)-2-azetidinylmethoxy]pyridine}, \text{ and } 10 \text{ selected } \text{N}-\text{substituted-azetidinyl} \text{ and pyrrolidinyl closely related } ^{18}\text{F}\text{-fluoropyridinyl derivatives (including } [N’-\text{aromatic/A}lphatic]-\text{thioureas, } -\text{ureas, and } -\text{amides}). \text{ The in vitro BBB model, a new coculture system of primary human brain endothelial cells and astrocytes, was used to measure the permeability coefficient for each compound. Dynamic PET studies were performed in rats with the same compounds, and a two-compartment model analysis was used to calculate their in vivo permeability coefficients. The 11 derivatives differed in their degree of BBB passage and transport mechanism. The analysis of PET data showed a significant cerebral uptake for six derivatives, for which the in vivo evaluation indicated active influx or free diffusion. Five derivatives displayed low in vivo cerebral uptake, in agreement with the observation of an in vitro active efflux. Overall, there was a remarkable correlation between the in vitro and in vivo permeability coefficients \((r = 0.99))\). This double study proves a close correlation between the assessment of the BBB passage in vitro and in vivo. The in vitro model of human BBB offers the possibility of subtle discrimination of various BBB permeability degrees and transport mechanisms. Conversely, small animal PET imaging appears suitable to screen directly in vivo brain targeting of drugs or radiopharmaceutical candidates.

In mammals, the presence of tight junctions connecting the endothelial cells of the brain vessels creates a blood-brain (BBB) that limits, to a considerable extent, the delivery of systemically administered drugs to the central nervous system (CNS). In addition, specific metabolizing enzymes and efflux pumps located within the endothelial cells actively degrade or reject exogenous molecules out of the brain (Schinkel et al., 1994; Huai-Yun et al., 1998; el-Bacha and Minn, 1999; Bendayan et al., 2002).

As a consequence, the development of drugs targeting the
CNS requires a precise knowledge of their brain penetration and, ideally, this information should be obtained as early as possible. This is a major challenge for the design of CNS drugs since 1) prediction of BBB passage from the chemical structures is largely unreliable and 2) considering the new compounds for which toxicological knowledge is lacking, it is not possible to test drug penetration directly in humans in vivo. Hence, testing the brain passage of drug candidates of pharmaceutical importance for CNS diseases relies on surrogate estimates of blood-to-brain passage established on in vitro and in vivo models of the BBB.

In vitro models are based on the reconstitution of the BBB by cell cultures of noncerebral peripheral endothelial cell lines, immortalized rat brain endothelial cells (Begley et al., 1996), primary cultured bovine, porcine or rat brain capillary endothelial cells (Audus and Borchardt, 1986; Van Bree et al., 1988; Hughes and Lantos, 1989; Weber et al., 1993; Franke et al., 1999), and of cocultures of primary brain capillary cells with astrocytes or astrocyte-conditioned medium (Purridge et al., 1990; Rubin et al., 1991; Dehouck et al., 1992; Gaillard et al., 2001; Mégard et al., 2002; Jeliazkova-Mecheva and Bobilya, 2003; Parkinson et al., 2003). Coculture systems produce monolayers with tight junctions joining the endothelial cells in which the polarity of transport can be evaluated by measuring the passage from apical to basolateral surface or vice versa. Despite their resemblance with the BBB, in vitro models must be carefully assessed for their capacity to describe accurately the passage of drugs into the CNS in vivo.

Alternatively, several in vivo experimental setups have been used to estimate the BBB passage of drugs directly in laboratory animals. In vivo transport across the BBB was first studied in the 1960s using the early indicator diffusion method (Crone, 1963). Other in vivo techniques were later proposed: brain uptake index measurement (Oldendorf, 1970), in situ brain perfusion or brain efflux index methods (Takasato et al., 1984; Kakee et al., 1996), autoradiography, and intracerebral microdialysis (Elmquist and Sawchuk, 1997). Although they require various levels of equipment, technical expertise, and mathematic modeling, all of these in vivo methods have important limitations, notably their invasiveness, which may lead to nonphysiological BBB passage. In contrast, functional imaging with positron emission tomography (PET) is a noninvasive imaging technique increasingly used in drug discovery (Gupta et al., 2002; Laruelle et al., 2003; Wong and Pomper, 2003), and it has been demonstrated to be applicable to the non invasive measurement of brain uptake index in baboons (Dishino et al., 1983). PET is today the most advanced technology to obtain biochemical information, such as glucose metabolism, blood flow, and distribution of receptors, enzymes, and neurotransmitters directly in vivo, because it is noninvasive, rapid, and repeatable and offers very high sensitivity. Thus, it allows monitoring of the whole pharmacokinetic time course in physiological conditions on the same animal. Brain kinetics can be analyzed by compartmental modeling, which allows the calculation of the relevant rate constants that describe the BBB passage (Koepe et al., 1990; Wiesel et al., 1991; Hendrikse et al., 2001).

Up to date, all models, whether in vitro or in vivo, remain mere approximates of the complex human BBB and their relevance to the real-life situation must be carefully controlled. An interesting way to do so is to cross-compare the BBB passage of a series of compounds evaluated with both in vitro and in vivo models alongside each other. This enables cross-correlatives of the in vitro and in vivo pharmacokinetic data and the assessment of the predictive power of both tests.

The present work reports for the first time the evaluation of the BBB permeabilities of a series of compounds studied in vitro using a new human BBB coculture system and in vivo with quantitative PET imaging in rats. The tested compounds are 11 selected fluorine-18-labeled fluoropyridinyl derivatives, including 1) the radiopharmaceutical \([^{18}\text{F}]\text{F-A-85380}\), a selective and high-affinity radioligand that is currently used for the study of the \(\alpha_2\beta_2\) nicotinic acetylcholine receptors in the human brain with PET (Dollé et al., 1998, 1999; Bottlaender et al., 2003; Kimes et al., 2003) and 2) 10 selected N-substituted-azetidinyl and -pyrrolidinyl closely related \([^{18}\text{F}]\text{fluoropyridinyl derivatives (including \([N-\text{aromatic/aliphatic}\)-thioureas, -ureas, and -amides) named \([^{18}\text{F}]\text{FPy}\) 01 to 10. Comparative measurements of the BBB passage of the complete series of compounds, estimated from the in vitro human BBB model and from the in vivo PET technique, are discussed.

Materials and Methods

Labeled Derivatives. \([^{18}\text{F}]\text{F-A-85380}\) was prepared as described previously (Dollé et al., 1999). In brief, \([^{18}\text{F}]\text{F-A-85380}\) was synthesized by a no-carrier-added nucleophilic aromatic substitution by \([^{18}\text{F}]\text{K}_{222}\) complex with 3-(2(S)-N-(tert-butoxycarbonyl)-2-aze
dimidinylmethoxy)pyridin-2-yltrimethylammonium trifluoromethane-sulfonate as a highly efficient labeling precursor followed by trifluoroacetic acid removal of the Boc protective group and HPLC purification (total synthesis time: 50–53 min from the end of cyclotron fluoride-18 production; radiochemical yields, with respect to initial \([^{18}\text{F}]\text{fluoride ion radioactivity, 68–72 (decay-corrected) and 49–52% (nondecay-corrected); specific radioactivities at the end of the synthesis: 2–5 Ci/\mu\text{mol (74–185 GBq/\mu mol).}}

The 10 selected N-substituted-azetidinyl and -pyrrolidinyl closely related derivatives \([^{18}\text{F}]\text{FPy}\) 01 to 10 (Fig. 1) were prepared using a similar chemical approach to the one described above for the preparation of \([^{18}\text{F}]\text{F-A-85380}\) followed by 1) condensation reaction of the cyclic amine with the appropriate commercially available isothiocyanate, isocyanate, or acyl chloride and 2) final HPLC purification to give the expected corresponding \([N-\text{aromatic/aliphatic}\)-thioureas, -ureas, and -amides.

Measurement of Octanol: Phosphate Saline Buffer Partition Coefficient. Five hundred microliters of octanol were added to each of the fluoride-18-labeled fluoropyridinyl derivatives (3700 kBq) diluted in 500 \(\mu\text{L of Dulbecco’s phosphate-buffered saline, pH 7.4 (PBS; Invitrogen, Carlsbad, CA).}}

The vial was shaken for 3 min, and the resulting homogenate was centrifuged for 5 min at room temperature. An aliquot (100 \(\mu\text{L of each phase was counted for radioactivity in a calibrated \(\gamma\)-counter (COBRA Quantum; PerkinElmer Life and Analytical Sciences (Boston, MA).}}

In Vitro Human BBB Model. The BBB model was a coculture of primary human brain endothelial cell(s) (BEC) and primary human astrocytes (HA) as described previously (Mégard et al., 2002). The isolation of these cells was essentially based on the methods described by Deli and Joo (1996). Immunocytochemistry specific for the HA and the BEC was conducted in order to check the purity of the cultures. BECs were cultured twice with PBS and incubated within 3 h at 37°C with 1-methyl-[\(^{14}\text{C}]1,1’-dioctadecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate-acetylated low-density lipoprotein (Di-acyl-LDL) (10 \(\mu\text{g·mL}^{-1}\) (Sigma-Aldrich, St Louis, MO) and then washed three times with PBS and fixed with paraformerdehyde in
through the monolayer of the paracellular reference marker [14C]glucose (12.95 MBq · mol⁻¹) (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as described previously (Megard et al., 2002).

**In Vivo Drug Transport Study.** Just before the transport experiments, the astrocytes were removed from the basal compartment and the media from both apical and basal compartments were replaced by brain microvascular endothelial cell-specific medium and astrocyte-specific medium, respectively.

Experiments were made in triplicate for each of the fluoropyridinyl derivatives. The radiolabeled compound (185 kBq) was introduced in the donor chamber (either the apical or the basal compartment). At various time points (5, 10, 15, 20, 30, and 40 min) after the addition of the radiolabeled compound, aliquots of the medium were removed from the acceptor chamber (basal or apical compartments, respectively) for radioactivity counting and were replaced by fresh medium.

**Data Analysis.** Permeability calculations were performed as described by Partridge et al. (1990). The fraction of radioactivity transported from the donor to the acceptor chamber at each time point was multiplied by the volume of the donor chamber to give the equivalent volume cleared at a given time point. The volume cleared was plotted versus time (clearance curve) and analyzed by linear regression. The clearance curve was linear up to 40 min for all derivatives. The slope of the clearance is the permeability coefficient PS expressed in microliter per minute⁻¹, where P is the permeability in millimolar per minute⁻¹ and S the filter area in squared millimeter. PS was measured both from the wells containing the filter plus endothelial cells (PSout) and from the wells with filter alone (PSf). The PS value strictly due to the endothelial monolayer is called PSe and was calculated as follows.

\[
\frac{1}{PSe} = \frac{1}{PStot} - \frac{1}{PSf}
\]

PSe was measured both from the apical to basal compartment (PSe-in) and from the basal to apical compartment (PSe-out), and the PSe-out/PSe-in ratio (Q ratio) was calculated.

**In Vivo PET Imaging.** All of the animal experiments were conducted in agreement with the “Principles of Laboratory Animal Care” (National Institutes of Health Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication 85-23, 1985). Male Wistar rats (200–250 g; IFFA-Credo, L’Arbresle, France) were anesthetized (isoflurane/oxygen, 5% for induction and 3% thereafter) and catheterized in the tail vein. A transmission scan with a Germanium-68 source was done for attenuation correction. The [18F]fluoropyridinyl derivative (14.8–15.5 MBq) was intravenously injected simultaneously into four anesthetized rats, and PET imaging was performed within 60 min with the EXACT HR+ camera (Siemens Medical Solutions, Knoxville, TN) with increasing time frames starting with 24 frames of 10 s followed by 18 frames of 20 s, 20 frames of 30 s, and finally 40 frames of 60 s. The raw data were reconstructed using an ordered subset expectation maximization weighted attenuation (OSEM-WA) with six iterations and eight subsets including a Fourier rebinning.

To determine the pharmacokinetics of the [18F]fluoropyridinyl derivatives in the brain, cerebral PET images were analyzed by drawing volumes of interest on the brain area visualized on eight consecutive sections by comparing the complete brain volume of the animals. For comparison, all values of radioactivity concentrations were normalized by the injected dose and expressed as percentage of the injected dose per volume of tissue (% ID · cm⁻³).

**Arterial Kinetics and Metabolism.** To estimate the input function, 14.8 to 15.5 MBq (200–250 μl) of each [18F]fluoropyridinyl derivative were injected to two animals. Arterial blood samples (20 to 30 μl) were removed at 1, 3, 6, 9, 12, and 15 s and then every 5 s up to 2 min and finally every 10 min until 1 h postinjection. Whole-blood radioactivity was measured in a calibrated γ-counter (COBRA II; PerkinElmer Life and Analytical Sciences).
To estimate the metabolism, 26.2 to 30.6 MBq (200–250 µl) of each [18F]fluoropropidin derivative were injected into a rat and 1 ml of arterial blood was removed at 5 min postinjection. Radio HPLC was used to measure the proportion of the intact compound in plasma.

**In Vivo Data Analysis.** The whole-blood kinetics and the brain kinetics obtained from PET data were analyzed according to a two-compartment model (Fig. 2). The operational equation of this model was as follows,

$$dC_b(t)/dt = k_1 \times C_a(t) - k_2 \times C_b(t)$$  

where $k_1$ is the influx rate constant expressed in milliliter of blood per cubic centimeter of tissue per minute, $k_2$ is the efflux rate constant (minute$^{-1}$), $C_b(t)$ is the concentration of radioactivity in the brain, and $C_a(t)$ is the arterial input function.

The biomedical software PMOD (PMOD Technologies, Zurich, Switzerland) was used to calculate the pharmacokinetic parameters $k_1$ and $k_2$ from each individual experimental arterial kinetic data (kilobecquerel per milliliter and the average brain kinetic data (kilobecquerle per centimeter$^{-3}$). The fit quality is estimated by the software and is reflected by the variation coefficients on $k_1$ and $k_2$ (see Table 2). The distribution volume (DV) is the ratio $k_1/k_2$ expressed in milliliter of blood per cubic centimeter of tissue. These calculations take into account the cerebrovascular blood volume, which was approximated to be 0.1 ml for the rat. $k_1$ reflects the rate of drug absorption into the brain, also called clearance of the drug,

$$k_1 = Q \times E$$  

where $Q$ is the blood flow (milliliter per minute$^{-1}$) and $E$ is the extraction ratio, a unitless quantity reflecting the proportion of drug and $k_2$ (see Table 2). The distribution volume (DV) is the ratio $k_1/k_2$ expressed in milliliter of blood per cubic centimeter of tissue. These calculations take into account the cerebrovascular blood volume, which was approximated to be 0.1 ml for the rat. $k_1$ reflects the rate of drug absorption into the brain, also called clearance of the drug,

$$E = 1 - \exp(-PS/Q)$$  

where $PS$ is the permeability $\times$ surface area product expressed in milliliter of blood per minute per cubic centimeter of tissue, $P$ is the permeability coefficient expressed in centimeter per minute$^{-1}$ and $S$ is the total area of the capillary bed expressed in square centimeters of capillary per cubic centimeter of tissue. Combining eqs. 4 and 5 yields eq. 6,

$$k_1 = Q(1 - \exp(-PS/Q))$$  

Hence, $PS$ can be deduced from eq. 6,

$$PS = -Q \times \ln(1 - k_2/Q)$$  

$Q$ was approximated to be 1.3 ml $\cdot$ min$^{-1}$ as previously published (Davies and Morris, 1993).

**In Vitro and In Vivo Correlation Study.** When attempting to measure drug transport across the BBB beyond the choice of the most relevant experimental models, it is essential to determine which in vitro and in vivo parameters are 1) the most significant and 2) can be used for comparison in the respective models. The present study focused on the transport stage without taking into consideration the fate of compounds once they have entered the brain. Using a two-compartment model to calculate the pharmacokinetic parameters of the BBB passage in vivo (i.e., the influx rate constant $k_1$ and the efflux rate constant $k_2$) allowed a direct comparison of the clearance of the compound from blood to brain (milliliter/minute/cubic centimeter of tissue), the same parameter being measured in the coculture-based model from the apical to the basal compartment (microliter/minute). The in vitro PS-in and the in vivo PS were both calculated from their respective measured clearances, so the comparison of these two parameters should be very accurate.

**Statistical Analysis.** Statistical analysis was performed using the Prism 3.0 program (GraphPad Software, Inc., San Diego, CA). Regression lines were calculated, and correlation was estimated by the two-tailed nonparametric Spearman test.

**Results**

**In Vitro Human BBB Model.** Specific immunocytochemical labeling with anti-GFAP mAb on astrocytes and Dil-acyl-LDL uptake in BEC or BEC stained for CD31 are illustrated in Fig. 3, A to C, respectively. In the coculture model, BEC formed a confluent monolayer within 15 days. Figure 3D shows the continuous network of labeled claudin-5, demonstrating that the cocultured endothelial monolayer displayed well developed tight junctions in the in vitro human BBB model.

**In Vitro Drug Transport Study.** Before the in vitro human BBB model was used to measure the BBB passage of the [18F]fluoropropidin derivatives, the tightness of the BEC monolayer was checked by assessing the permeability of [14C]sucrose. The very low permeability coefficient measured for [14C]sucrose (PS-in = 1.0 ± 0.15 µl $\cdot$ min$^{-1}$) demonstrated the integrity of the BEC monolayer. The permeability of the BEC monolayer was calculated from their respective measured clearances, so the comparison of these two parameters should be very accurate.

![Fig. 2. Two-compartment model. $k_1$ is expressed in milliliter of blood per cubic centimeter of tissue per minute, $k_2$ is expressed as minute$^{-1}$, $C_b(t)$ is the concentration of radioactivity in the brain, and $C_a(t)$ is the arterial input function.](image-url)

![Fig. 3. Fluorescence photomicrographs. A, primary human astrocytes stained for GFAP (secondary antibody anti-mouse IgG, fluorescein isothiocyanate-labeled). Nuclear staining with 4',6-diamino-2-phenylindole (blue). Scale bar = 25 µm. B, nonconfluent human brain microvascular endothelial cell component of the coculture BBB model stained with the fluorescent probe Dil-acyl-LDL (red). This fluorescent marker accumulates around endothelial cells nuclei. Nuclear staining with 4',6-diamino-2-phenylindole (blue). Scale bar = 40 µm. C, confluent human brain microvascular endothelial cell component of the coculture BBB model stained with the fluorescent probe Dil-acyl-LDL (red). This fluorescent marker accumulates around endothelial cells nuclei. Nuclear staining with 4',6-diamino-2-phenylindole (blue). Scale bar = 40 µm. D, confluent BEC monolayers grown on filters and stained for tight junction protein claudin-5 (secondary antibody anti-mouse IgG, Alexa Fluor 488-labeled). Stack size: x = 230.3 µm; y = 220.3 µm.](image-url)
of the $^{18}$F-fluoropyridinyl derivatives was then measured on the ways in and out (PSe-in and PSe-out), and the Q ratio was calculated (Table 1).

The in vitro model allows the assessment of the BBB permeability of the drug in both directions (influx and efflux) independently. Compounds that display efflux at least twice that of the influx (Q ratio above 2) are considered to undergo an effective efflux, and compounds that display efflux less than or equal to the influx (Q ratio below or close to 1.0) are considered to undergo an effective influx or a free diffusion, respectively. Effective efflux and influx imply the involvement of an active transporter.

In this study, the Q ratio ranged from 0.5 to 4.0. $^{18}$F-FPy 01, 03, 07, and 09 showed a Q ratio below 0.8, suggesting that these compounds cross the BBB by active transport. Two derivatives displayed a Q ratio close to 1 ($^{18}$F-A-85380 and $^{18}$F-FPy 08), suggesting that these compounds cross the BBB by free diffusion. Five derivatives showed a Q ratio above 2.0 ($^{18}$F-FPy 02, 04, 05, 06, and 10), suggesting that these compounds are subject to active efflux.

The metabolism analysis showed that all compounds were essentially intact in blood 5 min after injection (intact compound $\geq$98% of total blood radioactivity).

Whole-body PET images acquired over 2 min after the intravenous injections of the radiolabeled molecules are shown in Fig. 4. This early time point, high activity in the brain was clearly seen for derivatives $^{18}$F-FPy 01, 03, 08, 09, and 10 and low activity was evidenced for derivatives $^{18}$F-F-A-85380 and $^{18}$F-FPy 02, 05, and 06.

Figure 5 shows the percentage of the injected dose taken up per cubic centimeter of brain from 10 s to 3 min after the i.v. injection. The parent compound $^{18}$F-F-A-85380 showed an early and fugacious peak of 0.26 $\pm$ 0.02% ID·cm$^{-3}$ at 20 s after i.v. injection, after which the cerebral uptake gradually increased to a plateau reached at 40 min postinjection (0.5 $\pm$ 0.03% ID·cm$^{-3}$). These findings are in agreement with the binding of this compound to the cerebral $\alpha_{1}\beta_{2}$ nicotinic receptors and with reported biodistribution studies in rodents (Dollé et al., 1999). In contrast, the derivatives peaked between 0.4 and 1.8% ID·cm$^{-3}$ in a time range of 20 to 50 s, after which all derivatives showed a decrease in their brain activity up to 60 min, reflecting the lack of cerebral accumulation.

The pharmacokinetics of the BBB passage were analyzed according to a two-compartment model (arterial blood and brain tissue) separated by the BBB. The in vivo cerebral and whole-blood kinetics (Figs. 5 and 6) were analyzed according to this model, and the $k_{1}$, the $k_{2}$, the DV, and PS were calculated (Table 2). $k_{1}$ and $k_{2}$ ranged from 0.10 to 2.00 ml·cm$^{-3}$·min$^{-1}$ and from 0.04 to 2.08 min$^{-1}$, respectively.

### Table 1

Molecular weights, octanol/phosphate-buffered saline (pH 7.4) partition coefficients (log D), PSe-in and PSe-out, and Q ratio measured with the in vitro human BBB model for the 11 compounds studied.

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Mol. Wt.</th>
<th>Log D (pH 7.4)</th>
<th>PSe-in</th>
<th>PSe-out</th>
<th>Q ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-A-85380</td>
<td>182.2</td>
<td>1.49</td>
<td>5.68</td>
<td>4.48</td>
<td>0.9</td>
</tr>
<tr>
<td>FPy 01</td>
<td>317.4</td>
<td>1.23</td>
<td>10.39</td>
<td>6.46</td>
<td>0.6</td>
</tr>
<tr>
<td>FPy 02</td>
<td>281.3</td>
<td>0.63</td>
<td>10.04</td>
<td>36.85</td>
<td>3.7</td>
</tr>
<tr>
<td>FPy 03</td>
<td>286.3</td>
<td>1.93</td>
<td>59.42</td>
<td>34.26</td>
<td>0.6</td>
</tr>
<tr>
<td>FPy 04</td>
<td>266.3</td>
<td>1.57</td>
<td>12.87</td>
<td>51.38</td>
<td>4.0</td>
</tr>
<tr>
<td>FPy 05</td>
<td>585.6</td>
<td>1.36</td>
<td>3.34</td>
<td>8.12</td>
<td>2.4</td>
</tr>
<tr>
<td>FPy 06</td>
<td>321.4</td>
<td>1.95</td>
<td>6.47</td>
<td>24.87</td>
<td>3.8</td>
</tr>
<tr>
<td>FPy 07</td>
<td>337.5</td>
<td>1.15</td>
<td>19.58</td>
<td>5.43</td>
<td>0.3</td>
</tr>
<tr>
<td>FPy 08</td>
<td>321.4</td>
<td>1.74</td>
<td>16.22</td>
<td>12.86</td>
<td>0.8</td>
</tr>
<tr>
<td>FPy 09</td>
<td>315.3</td>
<td>1.76</td>
<td>8.70</td>
<td>3.91</td>
<td>0.5</td>
</tr>
<tr>
<td>FPy 10</td>
<td>295.4</td>
<td>1.74</td>
<td>1.68</td>
<td>3.67</td>
<td>2.2</td>
</tr>
</tbody>
</table>
F-A-85380 displayed the smallest values for $k_1$ and $k_2$ (0.10 ml$^{-1}$cm$^{-3}$ min$^{-1}$ and 0.04 min$^{-1}$, respectively), indicating a very slow entry and an even slower efflux. These results are in agreement with the binding of this compound to the cerebral nicotinic receptors, which restricts the efflux, and with previous PET studies in baboons and humans (Valette et al., 1999; Bottlaender et al., 2003; Kimes et al., 2003). In Vivo versus in Vitro Studies. $[^{18}F]$F-A-85380 displayed an in vitro Q ratio close to 1 (Q = 0.9), indicating a free diffusion into the brain by passive influx, whereas in vivo, the PET analysis demonstrated a gradual cerebral uptake (DV = 2.33), which reached a plateau at 40 min postinjection. Those results indicated that $[^{18}F]$F-A-85380 enters the brain by a passive influx and is not subject to efflux out of the brain.

When comparing the in vitro Q ratio with the in vivo DV (Fig. 7), the compounds were separated in two groups; derivatives with an in vitro Q ratio above 2.0 (active efflux) displayed low in vivo DV (below 0.6) corresponding to a slight cerebral entry ($[^{18}F]$FPy 02, 04, 05, 06, and 10). Conversely, derivatives with an in vitro Q ratio close to or below 1.0 (free diffusion or active influx, respectively) displayed high in vivo DV (above 0.6), reflecting their cerebral penetration ($[^{18}F]$F-A-85380 and $[^{18}F]$FPy 01, 03, 07, 08, and 09). Moreover, the in vitro permeability coefficient PSe-in determined with the in vitro human BBB model showed a highly significant correlation with the in vivo PS calculated from the in vivo cerebral and plasma kinetics ($r^2 = 0.985; p < 0.001$; Fig. 8).

Discussion

Physicochemical characteristics of drugs, such as molecular size and weight, lipophilicity, affinity, and selectivity for the target site or the metabolic profile are generally inadequate to predict with accuracy their passage through the human BBB (Tanaka and Mizojiri, 1999; Laruelle et al., 2003; Wong and Pomper, 2003). This was confirmed in the present study in which the tested compounds were in a relatively narrow range of molecular weights (266.3 to 585.6 g·mol$^{-1}$) and lipophilicities (log D, 0.63 to 1.95). Neither of these characteristics correlated with the measured permeability coefficients ($r^2 = 0.054$ and 0.068, respectively) nor was any relationship found between the biochemical structure and the BBB permeability. These results highlight the necessity to screen compounds intended to target the brain beyond their biochemical characteristics and to actually assess their permeability across the BBB either in vitro or in vivo.

In vitro models of the BBB have the advantage to be based on cell cultures that are relatively easy to implement and standardize and avoid the use of animals. However, a major factor that may restrict brain uptake is the presence of efflux...
pumps within the endothelial cells, such as the P-glycoprotein (P-gp), the breast cancer resistance protein, and the multidrug resistance protein, which act to keep nonessential molecules out of the brain (Schinkel et al., 1994; el-Bacha and Minn, 1999; Bendayan et al., 2002). Among the many in vitro models described, only a few have demonstrated the presence and functionality of these efflux transporters (Bendayan et al., 2002). In addition, with the exception of the recently published porcine and rat models (Jeliazkova-Mecheva and Bobilya, 2003; Parkinson et al., 2003), most coculture-based models display interspecies or age differences between the two cell types. This may not be relevant to achieve correct function, because brain uptake may be restricted by the activity within the cerebrovascular endothelial cells of several families of metabolizing enzymes, which are known to be species-specific (el-Bacha and Minn, 1999).

The new coculture-based model of human BBB presented here consists of primary cultures of human brain capillary endothelial cells, cocultivated with primary cultures of human astrocytes (Mégard et al., 2002). It is worth noting that this is the first establishment of a human astrocyte/endothelial cell coculture BBB model. The advantages of this system are that 1) it is made of primary culture cells, 2) it avoids species, age, and interindividual differences because the two cell types are removed from the same person, and 3) it has been shown to express functional efflux transporters, such as P-gp (Mégard et al., 2002), multidrug resistance protein-1, and breast cancer resistance protein (data not shown).

Direct in vivo evaluation of drug passage of the BBB is ideal, but considering the new compounds for which toxicological knowledge is lacking, it is limited to laboratory animals. Rodent models provide the best first guess provided that species differences are accounted for when necessary. This raises the issue of the relevance of animal BBB to human BBB. In this respect, even though species differences in metabolizing enzymes exist (Schellens et al., 2000), various rat membrane-bound transporters, among which is P-gp, have been demonstrated to have a strong homology with the human proteins (Schinkel et al., 1994).

Moreover, recent progresses in molecular imaging techniques now allow the assessment of drug distribution in vivo noninvasively. This is particularly the case with PET, a whole-body-imaging technique that can measure quantitatively in real time the tissular distribution of positron-emitter-labeled compounds. Progress in spatial resolution and in labeling techniques now permit dynamic quantitative measurements of drug pharmacokinetics in the organs of small laboratory animals.

Whether in vitro or in vivo, the validity of models pretending to describe such a complex system as the BBB must be tested with a series of compounds with different BBB permeabilities. It is classic, in situations in which a physiological mechanism is not directly measurable, to compare the results obtained by two models in a series of independent measurements. In particular, the prime importance of cross-correlation in vitro and in vivo pharmacokinetic data to validate experimental models and to assess the predictive power of the techniques has long been recognized. Accordingly, several studies conducted in vitro and in vivo BBB transport evaluations alongside each other (Pardridge et al., 1990; Dehouck et al., 1992; Pirro et al., 1994; Friebe et al., 2000; Lundquist et al., 2002). An in vitro/in vivo correlation study using another imaging technique has been previously published (Pirro et al., 1994). The BBB permeability was evaluated with both an in vitro bovine BBB model and the in vivo single-pass brain extraction measured by single photon emission computerized tomography. Despite a good preliminary in vitro/in vivo correlation for seven compounds, the authors found that several 99mTc complexes displayed no concordance between in vitro permeability indices and in vivo single-pass cerebral extraction. Pardridge et al. (1990) compared an in vitro BBB model consisting of bovine brain capillary endothelial cell monolayer and the internal carotid artery perfusion/capillary depletion in vivo method. The study demonstrated a correlation between the in vitro and in vivo ln permeability × surface area products ($r = 0.85$). However, overestimation of PSe values for lipid-mediated transport and underestimation of PSe values for carrier-mediated transport were observed with this in vitro model. These findings were attributed to the loss of expression of BBB-specific proteins in endothelium cultured in the absence of astrocytic trophic factors, which are normally secreted due to the close apposition of astrocytes on brain capillary endothelial cells in vivo (DeBault et al., 1980). To thwart this problem, the coculture of brain capillary endothelial cells and astrocytes was pioneered by Dehouck et al. (1990) and it was compared with the intracarotid injection in vivo method. The results showed a correlation ($r = 0.88$) between the in vitro and in vivo brain extraction values (Dehouck et al., 1992).

The same group demonstrated the superiority of the coculture system by comparing the in vivo brain extraction using the Oldendorf method and the in vitro permeability coefficient measured either with a primary culture of brain microvessel endothelial cells or with a coculture of brain capillary endothelial cells and astrocytes. In both cases, the in vivo and in vitro permeability values had correlated, but the coculture-based model displayed better indications than the endothelial cells monolayer ($r = 0.90$ and 0.96, respectively).

This strategy was followed in the present study in which the BBB passage of a series of compounds was evaluated with both in vitro and in vivo models alongside each other, enabling cross-correlations of the in vitro and in vivo pharmacokinetic data and the assessment of the predictive power of both tests. The present work reports for the first time the evaluation of the BBB permeabilities of a series of compounds studied correlatively in vitro using a human BBB coculture system and in vivo with whole-body PET scanning in rats. This study intended to evaluate whether the in vitro BBB model and the in vivo PET method could be able to prove any BBB passage differences related to small chemical structures variations.

The 11 tested derivatives differed in their degree of BBB passage and transport mechanism, and we cast new light on the close relationship between the in vitro and in vivo pharmacokinetic data. Two major observations should be pointed out. First, an in vitro/in vivo correlation was found between the in vitro Q ratio and the in vivo DV, allowing the discrimination between two groups of compounds. Five derivatives ($^{18}$F)FPy 02, 04, 05, 06, and 10) displayed low or absent in vivo cerebral penetration (DV <0.6), in agreement with an active efflux observed in vitro (Q >2). Conversely, for six derivatives ($^{18}$F)-A-85380 and $^{18}$F)FPy 01, 03, 07, 08, and 09) for which the PET analysis demonstrated a cerebral uptake (DV >0.6), the in vitro evaluation indicated either
free diffusion (Q < 1) (18)[F]-A-85380 and (18)[F]FPy 08) or active influx (Q < 1) (18)[F]FPy 01, 03, 07, and 09). Second, we established a strong correlation between the in vitro and in vivo permeability coefficients (r = 0.99).

Interestingly, the in vitro BBB model provides additional pharmacological information with regard to the in vivo evaluation. Indeed, the compounds with low in vitro Pse-in (18)[F]-A-85380 and (18)[F]FPy 10 and 05) cannot be well separated on the in vivo scale, because their in vivo PS are close to zero (0.10, 0.15, and 0.16, respectively). This may result from the restricted sensitivity of the in vivo method for the discrimination of low-entry capacity compounds in comparison with the very subtle in vitro evaluation. Furthermore, whereas in vivo cerebral pharmacokinetics supply global information on the degree and rate of BBB passage, the in vitro model is able to predict passive or active transport of molecules, suggesting for example the involvement of efflux transporters.

Even though PET cannot describe BBB passage with such accuracy, the correlation between the in vitro and the in vivo pharmacokinetic data demonstrates that imaging reliably predicted the BBB passage for this series of derivatives of the radiopharmaceutical (18)[F]-A-85380. If such a strong agreement between the in vitro and in vivo pharmacokinetic data can be confirmed with other classes of molecules with different hydrophobic and molecular size ranges, the PET-imaging method described here should prove useful for the rapid evaluation of the brain penetration of drug candidates.

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References


