Inhibition of P-gp activity at the primate blood-brain barrier increases the distribution of nelfinavir into the brain but not into the CSF

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Running title:

Inhibition of P-gp activity at the BBB in non-human primates

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P-gp, P-glycoprotein; BBB, blood-brain barrier; BCSFB, blood-cerebrospinal fluid barrier; CNS, central nervous system; PIs, protease inhibitors; HIV, human immunodeficiency virus; HAD, HIV-associated dementia; HAART, highly active anti-retroviral therapy; UPLC, Ultra performance liquid chromatography; BCRP, breast-cancer resistance protein; MRP, multidrug resistance-associated protein; AUC, area under the curve; PET, positron emission tomography.
ABSTRACT

P-glycoprotein (P-gp) expression at the rodent blood-brain barrier (BBB) limits the central nervous system (CNS) distribution of anti-HIV protease inhibitors (PIs). However, it is not clear if P-gp activity at the human BBB is as effective as in rodents in preventing the distribution of PIs into the CNS. If it is, inhibition of P-gp at the human BBB could increase the distribution of the PIs into the CNS and therefore their efficacy against HIV-associated dementia. Since the distribution of the PIs into the human brain cannot be directly measured we conducted studies in a more representative animal, the non-human primate. Specifically we investigated the distribution of nelfinavir (a PI and a P-gp substrate; 6 mg/kg iv) into the brain and CSF of non-human primates (cynomologus monkeys, M. fascicularis) in the presence and absence of the potent and selective P-gp inhibitor, zosuquidar, and if changes in brain nelfinavir concentration, after inhibition of P-gp, paralleled those in the CSF. Our data indicate that nelfinavir has poor penetration into the macaques’ brain and CSF, and P-gp inhibition at the BBB by zosuquidar enhanced nelfinavir’s distribution into the brain by 146-fold. However, nelfinavir’s concentration in the CSF was unaffected by co-administration of zosuquidar (p>0.05). In conclusion, P-gp inhibition at the non-human primate BBB significantly enhanced nelfinavir’s distribution into the brain and this effect was not observed in the CSF. Therefore, as is common in human studies investigating P-gp inhibition at the BBB, CSF concentration of a drug should not be used as a surrogate marker for brain drug concentration.
HIV-associated dementia (HAD) is a cognitive and motor disorder as a result of HIV-1 infection of the central nervous system (CNS) including the brain and cerebrospinal fluid (CSF) (Lee and Benadyan, 2004). The brain and CSF are considered as HIV reservoirs (McArthur JC et al., 1997). To effectively treat HAD sufficient concentrations of anti-HIV drugs in the brain and CSF should be attained. HIV protease inhibitors (PIs) are used as frontline therapy in the treatment of HIV patients and their inclusion in highly active antiretroviral therapy (HAART) regimen has significantly reduced plasma viral loads and the incidence of HAD. However, treatment failure continues (Dore et al. 1999). One reason for this failure is the poor penetration of the PIs into the CNS (Groothuis and Levy, 1997; Enting et al., 1998) because of the efflux of these drugs by P-glycoprotein (P-gp). P-gp is an ATP-binding cassette efflux transporter expressed at the blood-brain barrier (BBB) and blood-CSF barrier (BCSFB) (Sankatsing et al., 2004). PIs are substrates for P-gp which limits their penetration into the CNS resulting in lack of adequate treatment of HIV infection in the brain (Kim et al., 1998; Sankatsing et al., 2004). Accordingly, it is thought that inhibition of P-gp at the BBB may increase the CNS penetration of PIs and therefore increase their effectiveness in the treatment of HAD.

In humans, the distribution of the PIs into brain cannot be assessed directly. Therefore, the CSF compartment has been used as a surrogate marker of the concentrations of drugs in the brain. In humans, CSF concentration of PIs, such as nelfinavir, ritonavir and saquinavir, have been reported to be non-detectable or lower than those necessary to suppress HIV (Khaliq et al., 2000; Polis et al., 2003). If the CSF compartment is a surrogate marker of the brain concentrations of the PIs, these data are in agreement with those obtained in the P-gp knock-out mice that P-gp is effective in preventing the entry of the PIs into the brain. To test this widely held assumption, we conducted studies in a more representative animal, the non-human primate.
Specifically we investigated: 1) the distribution of nelfinavir (a PI and a P-gp substrate) into the brain and CSF of non-human primates after iv administration; 2) the effect of the potent and selective P-gp inhibitor, zosuquidar (LY-335979), on the distribution of nelfinavir into the brain and CSF; 3) if changes in brain nelfinavir concentrations, after inhibition of P-gp, paralleled those in the CSF.

Materials and Methods

Animal model. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Washington. Animals were housed and experiments were conducted under the auspices of the Institutional Animal Welfare. Under telezol/ketamine (5 mg/kg each) anesthesia, three adult macaques (M. fascicularis, 2.6-5.8 kg) were equipped with a lumbar catheter for CSF sampling and two venous catheters (saphenous or femoral veins) for blood sampling and drug administration, respectively. Immediately afterwards, in a crossover random fashion with wash-out period of 2 weeks, each animal was administered an iv bolus of either nelfinavir mesylate (6 mg/kg free base; Pfizer Inc, Groton, CT) alone or nelfinavir mesylate 5 min after administration of zosuquidar hydrochloride (3 mg/kg free base; zosuquidar was synthesized as described by Anderson et al, 2006). Nelfinavir mesylate and zosuquidar hydrochloride were dissolved in 10% sulfobutyl ether β-cyclodextrin (Sodium Salt, Cydex Inc., Lenexa, Kansas). Blood and CSF samples were collected prior to drugs administration and at 15 (blood only), 30, 60, and 90 min and 24 h after nelfinavir administration. To avoid subjecting the animals to prolonged sedation, they were allowed to recover from anesthesia between the 90 min and 24 h samples. For 24 h samples, the animals were anesthetized with telezol (5 mg/kg)
about 30 min prior to blood and CSF sampling. Plasma was harvested and all samples were stored at -70°C until analysis.

Nelfinavir distribution into the brain was examined in four adult macaques (M. fascicularis, 2.7-6.4 kg). Under telezol/ketamine anesthesia, two animals were administered nelfinavir (6 mg/kg, iv) alone and 2 were administered nelfinavir 5 min after zosuquidar administration (3 mg/kg, iv). Blood and CSF samples were collected prior to drug administration and 30-70 min post nelfinavir dose. Following blood and CSF sampling, animals were euthanized with pentobarbital (80-150 mg/kg, iv). Then, the brain was removed and divided along the midsagittal plane. White and gray matter of the right hemisphere were separated to determine nelfinavir and zosuquidar concentrations. All samples were stored at -70°C until analysis.

**Nelfinavir and zosuquidar analysis.** Nelfinavir and zosuquidar concentrations in plasma, CSF and brain homogenate (1:1 ratio, w/v, in PBS) were determined as described previously (Anderson et al., 2006) with minor modifications. Briefly, to 50 µl plasma, CSF or brain homogenate, the internal standard, saquinavir (100 ng/ml in methanol, 50 µl), ammonium hydroxide (0.75 M, 50 µl) and ethylacetate:acetonitrile (90:10, v/v, 1 ml) were added. After vortexing for 1 min, and centrifuging (3000 x g) for 10 min, the organic layer was collected. This extraction was repeated and the organic layers were combined. Samples were dried and reconstituted in mobile phase and 5 µl (brain or plasma extracts) or 30 µl (CSF extracts) were assayed as described below.

Nelfinavir, zosuquidar and saquinavir chromatographic separation was performed on a 50 x 2.1 mm Waters Aquity 1.7 µm BEH C18 column (Waters Corp., Milford, USA) using a Waters Aquity UPLC-MS system. The column was maintained at 50°C and eluted with a gradient program with initial conditions of A=95% and B=5% where A was 0.1% acetic acid in water and
B was 5 mM ammonium acetate (to minimize carry over) in methanol (0.3 ml/min). The gradient was as follows: from 0.5 -1.5 min B was increased to 100% and maintained at this condition until 2.5 min. Then, the mobile phase was returned back to its initial condition (2.5-3.0 min) and the column was allowed to equilibrate for 2 min. The analytes were detected by mass spectrometry using electrospray ionization (ESI) interface operated in positive mode. Instrument control and data acquisition were carried out by the MassLynx 4.0 software. Nitrogen was used as the cone and desolvation gas with flow rates of 14 and 1101 L/h, respectively. The capillary voltage was held at 3.0 kV and cone voltage was adjusted to maximize the response of the precursor ion (MH+) for each compound. The cone voltage was set at 40 V for nelfinavir, 25 V for zosuquidar and 35 V for saquinavir. The collision gas (argon) was turned on and the collision energy was optimized for the maximum production of product ions and was set at 30 eV for nelfinavir and saquinavir, while for zosuquidar, the collision energy was set at 20 eV. Source and desolvation temperatures of 120 and 400°C, respectively, were used. The compounds were detected and quantified by MS/MS in multiple-reaction monitoring (MRM) mode. The following transitions (precursor>product) were used for quantification; nelfinavir: 568>330, zosuquidar: 528>241, and saquinavir: 671>570.

Under these chromatographic conditions, the detector signal was linear with respect to the drug concentration over the ranges 0.5-25 ng/ml, 100-2500 ng/ml, and 10-1000 ng/ml of nelfinavir and 0.5-25 ng/ml, 50-1250 ng/ml, and 5-500 ng/ml of zosuquidar in CSF, plasma and brain, respectively. Samples reporting concentrations higher than these were diluted with the appropriate matrix to bring them into the linear range. Intraday precision and accuracy were examined at 2 different concentrations for each matrix. Quality control concentrations for nelfinavir were 3 and 15 ng/ml, 750 and 1750 ng/ml, and 150 and 600 ng/ml in CSF, plasma and
brain, respectively. For zosuquidar, these concentrations were 2 and 12 ng/ml, 300 and 700 ng/ml, and 75 and 350 ng/ml in CSF, plasma and brain, respectively. Intraday precision (CV%) for both compounds in CSF, plasma and brain were <14% and the accuracy ranged from 96-110% and 93-116% for nelfinavir and zosuquidar, respectively.

**Data analysis.** The plasma and CSF concentration (C) versus time (t) data of nelfinavir and zosuquidar were analyzed using the non-compartmental method. The extent of distribution of nelfinavir into the CSF was estimated as \( \frac{\text{AUC}_{\text{CSF}}}{\text{AUC}_{\text{plasma}}} \) where AUC is the area under the concentration-time curve. Also, the ratio of nelfinavir concentration in CSF (or brain) to plasma at all sampling times were calculated. All data are presented as mean ± SEM where applicable. The students paired t-test was used to determine any difference \((p<0.05)\) between the two groups.

**Results**

**Effect of zosuquidar on plasma and CSF concentrations of nelfinavir.** After administration of nelfinavir alone, plasma concentration of nelfinavir was measurable at 24 h in only one out of the 3 animals while it could be measured in the 3 animals administered both nelfinavir and zosuquidar. Therefore, AUCs from time 0 to 90 min \( (\text{AUC}_{0-90}) \) were used for comparisons. When compared with the control group, zosuquidar did not significantly change nelfinavir plasma \( \text{AUC}_{0-90} \) \((105 \pm 21 \mu\text{g.min/ml vs. } 92 \pm 14 \mu\text{g.min/ml, } p>0.05)\). Furthermore, nelfinavir CSF/plasma concentration ratio at 30, 60 and 90 min did not change significantly between the two groups \((p>0.3)\) (Table 1). Likewise, nelfinavir \( \frac{\text{AUC}_{\text{CSF}}}{\text{AUC}_{\text{plasma}}} \) ratio in the presence and absence of zosuquidar \((0.0014 \pm 0.0005 \text{ vs. } 0.0032 \pm 0.0019)\) was not significantly different \((p>0.4)\). The mean plasma concentrations of zosuquidar were: 548 ± 109, 485 ± 40, 410 ± 90 and 324 ± 116 ng/ml at 15, 30, 60 and 90 min, respectively.
Effect of zosuquidar on CSF/plasma and brain/plasma concentrations of nelfinavir. In animals treated with nelfinavir alone (n=2), CSF and plasma concentrations of nelfinavir were higher than in nelfinavir-zosuquidar group (n=2) (Table 2). However, the CSF/plasma ratios of nelfinavir concentrations were comparable in both groups and were 0.006 and 0.007 for nelfinavir alone (n=2) and nelfinavir-zosuquidar (n=2) groups, respectively (Table 2).

In the brain, nelfinavir and zosuquidar concentrations in the white and gray matter were measured in 3 different parts of the right hemisphere of the brain. Each sample was measured in triplicate and the average of nelfinavir concentration in the white and gray matter were compared. There was no significant difference in nelfinavir and zosuquidar concentrations in these two regions of the brain with coefficient of variation of the assayed concentrations ranging from 8-27% for nelfinavir and 8-16% for zosuquidar. Because nelfinavir and zosuquidar distribution in the white and gray matter across the right hemisphere was similar, an average of the concentrations in these two regions of the brain was used. Unlike the lack of effect on the distribution of nelfinavir into the CSF, zosuquidar caused a significant increase in the distribution of nelfinavir into the brain resulting in 146-fold increase in the C_{brain}/C_{plasma} ratio (Table 2). In contrast, zosuquidar caused a decrease in nelfinavir C_{CSF}/C_{brain} ratio (0.19 vs. 0.001) when co-administered with nelfinavir. The mean plasma, CSF and brain concentrations of zosuquidar were 929 ng/ml, 8.5 ng/ml, and 4816 ng/g, respectively (n=2).

Discussion

Recent studies in mice and rats have demonstrated that inhibition of P-gp at the BBB by zosuquidar results in a significant increase (26-37 fold) in the distribution of nelfinavir into the brain (Choo et al., 2000; Anderson et al., 2006). Since species can differ considerably in the
level of expression and activity of transporters, it is not clear if P-gp activity at the human BBB is as significant as that in the rodents. Since it is not ethically possible (except by PET imaging (e.g. Sasongko et al., 2005)) to measure the concentration of PIs in the human brain, the CSF concentration of these drugs has been used as a surrogate marker to indicate their brain concentration (Khaliq et al., 2000; van Praag et al., 2000). However, since the CSF compartment is a distinct compartment, it may not behave in parallel with the brain and therefore may not reflect the brain concentrations of the PIs. To address all the above issues, we used an animal model, the non-human primate, as a more representative model of P-gp activity at the human BBB. This model has previously been shown to be predictive of CSF distribution of drugs in humans (McArthur JC et al., 1997).

Because of the reported low concentrations of nelfinavir in human CSF (Polis et al., 2003; Sols et al., 2003), for this study we developed a highly sensitive UPLC method with a limit of quantification of 0.5 ng/ml of nelfinavir. In the absence of zosuquidar, nelfinavir’s poor distribution into the brain and the CSF was in agreement with studies in humans (Polis et al., 2003; Sols et al., 2003) and rodents (Choo et al., 2000; Anderson et al., 2006). As in humans, nelfinavir concentration in the macaque CSF was low and highly variable. On average, nelfinavir’s distribution into the CSF was found to be limited (<1% of plasma concentrations) and markedly lower than its distribution into the brain (4% of plasma concentrations). The later is comparable with the values reported in mice (6 ± 2%; Choo et al., 2000) and rats (6 ± 3%; Anderson et al., 2006) 2 h after drug administration.

Zosuquidar did not significantly alter nelfinavir concentrations (up to 90 min) in the macaque plasma or CSF (Table 1). However, it is possible that zosuquidar could have affected the overall clearance of nelfinavir as we could detect nelfinavir in the plasma at 24 h in the 3 animals who
were administered nelfinavir and zosuquidar but in only 1 out of the 3 animals administered nelfinavir alone. In contrast, pretreatment with zosuquidar significantly enhanced nelfinavir’s distribution into the macaque brain by ~146-fold. This dramatic increase in the distribution of nelfinavir into the macaque brain exceeded that produced in mice (37-fold; Choo et al., 2000) and rats (up to 26-fold; Anderson et al., 2006). Although this remarkable difference could be related to species differences, the magnitude of this difference should not be over-interpreted as none of these studies was conducted at steady-state concentrations. Therefore the magnitude of the change in nelfinavir’s distribution into the brain, in the presence and absence of a P-gp inhibitor, will be dependent on the distributional dynamics of the drug.

Based on the above data, we conclude that zosuquidar increased the brain distribution of nelfinavir by inhibiting P-gp at the BBB. This conclusion is reasonable as nelfinavir is a weak substrate of MRP1 (Bachmeier et al, 2005) and is not transported by BCRP (Gupta et al, 2004). In addition, zosuquidar is a potent and selective inhibitor of P-gp, is not a substrate of P-gp or BCRP (Shepard et al, 2003) and does not inhibit MRP1 or MRP2 (Dantzig et al., 1999).

In the absence of zosuquidar, nelfinavir concentrations in the CSF was found to be 19% of brain concentrations and this percentage significantly decreased to 0.1% by pretreatment with zosuquidar. This reduction was not related to changes in nelfinavir CSF concentrations but was due to an increase in the brain concentrations of nelfinavir caused by zosuquidar. These results provide compelling evidence that, as is customary in human studies investigating P-gp inhibition at the BBB, CSF concentrations of a drug cannot be used as a surrogate marker of the concentration of the drug in the brain. Thus, all human CSF studies claiming to do so should be viewed with caution. P-gp is located at the apical membrane of the choroids plexus (Sankatsing et al., 2004) and therefore effluxes drugs into the CSF. Inhibition of P-gp at the choroids plexus
will result in lower CSF concentrations of P-gp substrate drugs as described by Chen et al. (2006). These investigators reported that inhibition of P-gp at the choroids plexus by tamoxifen (a P-gp inhibitor) reduced paclitaxel concentrations in the CSF of brain tumor patients. Zosuquidar may have inhibited P-gp at the choroid plexus resulting in no change in CSF nelfinavir concentration. Different results were reported by Khaliq et al. (2000) and van Praag et al. (2000) who observed higher CSF concentrations of ritonavir and saquinavir (Khaliq et al., 2000) and indinavir (van Praag et al., 2000) in the presence of ketoconazole and ritonavir, respectively. However, these results were contradicted by Haas et al. (2003) who obtained serial CSF and plasma sampled from HIV-infected patients for AUC$_{CSF}$/AUC$_{plasma}$ evaluation. They reported that the primary mechanism of the increase in CSF indinavir concentration, when co-administered with ritonavir, was not due to P-gp inhibition but was due to increased plasma concentrations of indinavir due to hepatic CYP3A inhibition by ritonavir.

The zosuquidar dose utilized here was based on reported human plasma concentrations required to inhibit P-gp (Fracasso et al. 2004). The average plasma AUC$_{0-90}$ of zosuquidar was $30 \pm 7 \mu$g.min/ml, and its concentration in the brain after 30-75 min of its administration was 4816 ng/g. Given its high lipophilicity (logP = 4.5) and its inability to be effluxed by P-gp or BCRP (Shepard et al., 2003), zosuquidar’s significant concentrations in the brain was as expected and in agreement with other studies (Choo et al., 2000; Anderson et al., 2006). However, its low uptake into the CSF (~9 ng/ml), reported here for the first time, was surprising and below the reported in-vitro concentration required to inhibit 50% of P-gp activity (Ki ~ 32 ng/ml; Dantzig et al. 1996).

In conclusion, in non-human primates, inhibition by zosuquidar of P-gp enhanced the distribution of nelfinavir into the brain. Such inhibition is a potential strategy to enhance the
efficacy of the HIV protease inhibitors in the treatment of AIDS dementia. In addition, our studies provide compelling evidence that CSF drug concentrations, in studies investigating P-gp inhibition at the BBB, do not necessarily reflect drug concentrations in the brain. Thus, such studies reporting CSF concentrations to provide a quantitative measure of the distribution of drugs into the human brain must be viewed with caution.
References


Footnotes

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Table 1. Nelfinavir CSF/plasma concentration ratios at 30, 60 and 90 min after administration of a single dose of nelfinavir (6 mg/kg, iv) given alone or in combination with zosuquidar (3 mg/kg, iv), n=3 per group.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Animal</th>
<th>Nelfinavir alone</th>
<th>Nelfinavir + Zosuquidar</th>
<th>Fold ↑</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CSF (ng/ml)</td>
<td>Plasma (ng/ml)</td>
<td>C&lt;sub&gt;CSF/C_plasma&lt;/sub&gt;</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
<td>3.4</td>
<td>1660</td>
<td>0.0024</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.8</td>
<td>699</td>
<td>0.0026</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.1</td>
<td>1666</td>
<td>0.0007</td>
</tr>
<tr>
<td>60</td>
<td>1</td>
<td>4.2</td>
<td>822</td>
<td>0.0051</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.9</td>
<td>571</td>
<td>0.0016</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.9</td>
<td>1187</td>
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</tr>
<tr>
<td>90</td>
<td>1</td>
<td>2.2</td>
<td>705</td>
<td>0.0031</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.8</td>
<td>512</td>
<td>0.0035</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.5</td>
<td>835</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

* p>0.3
Table 2. Fold-increase in nelfinavir CSF and brain distribution when nelfinavir was administered alone (6 mg/kg, iv) or with zosuquidar (3 mg/kg, iv), n=2 per group.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Time (min*)</th>
<th>CSF (ng/ml)</th>
<th>Plasma (ng/ml)</th>
<th>Brain** (ng/g)</th>
<th>$C_{CSF}/C_{Plasma}$ Mean</th>
<th>$C_{Brain}/C_{Plasma}$ Mean</th>
<th>Fold ↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nelfinavir alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40-65</td>
<td>17</td>
<td>5073</td>
<td>16 ± 4</td>
<td>0.0034</td>
<td>0.0032</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>32-40</td>
<td>16</td>
<td>1857</td>
<td>157 ± 42</td>
<td>0.0086</td>
<td>0.006</td>
<td>0.0850 0.044</td>
</tr>
<tr>
<td>Nelfinavir + Zosuquidar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>34-75</td>
<td>6</td>
<td>663</td>
<td>4607 ± 345</td>
<td>0.0090</td>
<td>6.95</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>39-48</td>
<td>4</td>
<td>934</td>
<td>5473 ± 415</td>
<td>0.0043</td>
<td>5.86</td>
<td>0.2 146</td>
</tr>
</tbody>
</table>

* The first time indicates the time of CSF and blood sampling while the second time indicates the time of harvest of the brain tissue.

** Values represent the average of nelfinavir concentrations measured in different parts (n=6) of the brain ± SEM.