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## **Dependence of Nelfinavir Brain Uptake on Dose and Tissue Concentrations of the Selective P-gp Inhibitor Zosuquidar in Rats**

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**Running title:** Effect of Zosuquidar on Brain Uptake of Nelfinavir

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**List of nonstandard abbreviations:** ADC, AIDS dementia complex; CNS, central nervous system; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; MDR, multidrug resistance; BCRP, breast-cancer resistance protein

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## ABSTRACT

Most reverse transcriptase and protease inhibitors used in highly active antiretroviral therapy for treating human immunodeficiency virus (HIV) infections exhibit poor penetration into the brain, raising the concern that the brain may be a sanctuary site for the development of resistant HIV variants. This study explores the relationship between the dose and plasma and brain concentrations of zosuquidar and the effect of this selective P-glycoprotein inhibitor on CNS penetration of the HIV protease inhibitor nelfinavir maintained at steady-state by intravenous infusions in rats. Nelfinavir was infused (10 mg/kg/hr) for up to 10 hours with or without concurrent administration of an intravenous bolus dose of 2, 6, or 20 mg/kg zosuquidar given at 4 hours. Brain tissue and plasma were analyzed for both drug concentrations. Brain tissue:plasma nelfinavir concentration ratios (uncorrected for the vascular contribution) increased nonlinearly with zosuquidar dose from  $0.06 \pm 0.03$  in the absence of zosuquidar and  $0.09 \pm 0.02$  between 2-6 hours after a 2 mg/kg zosuquidar to  $0.85 \pm 0.19$  after 6 mg/kg and  $1.58 \pm 0.67$  after 20 mg/kg zosuquidar. Zosuquidar brain tissue:plasma concentration ratios exhibited a similar abrupt increase from  $2.8 \pm 0.3$  after a 2 mg/kg dose to  $\sim 15$  after the 6 mg/kg and 20 mg/kg doses. The apparent threshold in the plasma concentration of zosuquidar necessary to produce significant enhancement in brain uptake of nelfinavir appears to be close to the plasma concentrations associated with the maximum tolerated dose reported in the literature after repeated dosing of zosuquidar in patients.

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Despite the success of highly active antiretroviral therapy (HAART) in the treatment of human immunodeficiency virus (HIV) infected patients (Carpenter et al., 2000), incomplete long-term suppression of viral replication leading to the development of drug resistant viral strains continues to be a significant concern (Kirschner and Webb, 1997; Hirsch et al., 1998; Kepler and Perelson, 1998; Schragger and D'Souza, 1998; Winters et al., 2000; Solas et al., 2003). Most of the reverse transcriptase and protease inhibitors used in HAART exhibit poor penetration into the central nervous system (CNS) (Groothuis and Levy, 1997; Kim et al., 1998; Kravcik et al., 1999; Sawchuk and Yang, 1999; Lafeuillade et al., 2002). Consequently, the brain is particularly likely to serve as a sanctuary site for the development of resistant HIV variants (Pialoux et al., 1997; Cunningham et al., 2000; Smit et al., 2004). AIDS dementia complex (ADC) is an additional neurological complication of HIV replication in the CNS, affecting approximately 20 to 30% of patients with advanced HIV infection (Brew, 1999; McArthur et al., 2003), though its incidence has declined with the advent of HAART (Husstedt et al., 2002).

HIV protease inhibitors such as nelfinavir are significantly impeded in their transport across the blood-brain barrier by P-glycoprotein, a plasma membrane protein encoded by the multidrug resistance (MDR) gene that functions as an ATP-dependent efflux transporter (Kim et al., 1998; Lee et al., 1998; van der Sandt et al., 2001; Sankatsing et al., 2004). By inhibiting P-glycoprotein, it may be possible to increase protease inhibitor levels in the brain thereby reducing the role of the brain as a sanctuary site for viral replication and limiting the incidence of ADC. Previous studies, including our own, have demonstrated that potent P-glycoprotein inhibitors such as GF120918 and zosuquidar (LY-335979) can significantly enhance brain concentrations of protease inhibitors (Choo et al., 2000; Edwards et al., 2002; Savolainen et al., 2002; Edwards et al., 2005) and other P-glycoprotein substrates (Karszen et al., 2002; Kemper et al., 2004).

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In order to be therapeutically effective, P-glycoprotein inhibitors should be sufficiently potent to achieve inhibitory effects at non-toxic plasma concentrations and sufficiently selective for P-glycoprotein to minimize effects on overall drug pharmacokinetics. Zosuquidar, developed as a highly potent and selective inhibitor for P-glycoprotein (Dantzig et al., 1999), was selected as a clinical candidate for reversing anticancer drug resistance mediated by P-glycoprotein because it appeared to meet these criteria (Rubin et al., 2002). Indeed, Rubin et al. were able to demonstrate that biologically effective plasma concentrations of zosuquidar could be achieved with minimal toxicity and without significant alterations in the pharmacokinetics of doxorubicin. On the other hand, Kemper et al. concluded that the dose-limiting neurological toxicity that was observed by Rubin et al. occurs at plasma levels of zosuquidar that are insufficient to improve the penetration of paclitaxel into brain tumors by P-glycoprotein inhibition (Kemper et al., 2004).

This study explores further the relationship between the dose of zosuquidar, its plasma and brain concentrations, and its ability to enhance CNS penetration of the HIV protease inhibitor nelfinavir during infusions to steady-state in rats. We find a very steep dependence between the enhancement of brain uptake of nelfinavir and the dose and/or plasma concentration of zosuquidar suggestive of a threshold plasma concentration as suggested by Kemper et al (Kemper et al., 2004). Enhancement in brain uptake of nelfinavir coincides with an increase in the brain:plasma concentration ratio for zosuquidar.

## Materials and Methods

### Chemicals.

Nelfinavir free base was extracted from Viracept oral powder formulation (Pfizer, Inc., La Jolla, CA). The peak purity of each batch was determined by HPLC to be ~98-99%. Titration of a single batch in 90% methanol/water yielded an apparent purity based on equivalent weight of 102%.

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Zosuquidar (LY335979·3HCl, (2*R*)-anti-5-{3-[4-(10,11-difluoromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxy}quinoline trihydrochloride) was synthesized at the University of Kentucky following procedures adapted from the literature (Suzuki et al., 1997; Kroin and Norman, 1998). Its identity was established by HPLC comparison to authentic reference standard at Eli Lilly and Company (Indianapolis, IN). Peak purity was determined by HPLC to be 97% and titration in 90% methanol/water yielded an apparent purity based on equivalent weight of 99.5%.

### **Surgical Procedure and Preparation of Infusion Solutions.**

Female Sprague-Dawley rats were obtained from Charles River (Canada) and housed and cared for by the University of Kentucky Division of Laboratory Animal Research facilities. Animal procedures were performed using guidelines for the care and use of laboratory animals established by the University of Kentucky. Animals were anesthetized using 0.5 to 5% halothane in oxygen, to effect. Aseptic technique was used to implant catheters into the jugular and femoral veins as described by Waynforth and Flecnell (Waynforth and Flecnell, 1994). Hair was clipped from the inside of the left leg, the neck over the right jugular and from the back of the neck. All areas were cleaned with alcohol and painted with 10% povidone-iodine solution. A small incision was made in the skin and the appropriate vein was exposed. Using a 25-gauge needle, a hole was punctured in the vein. Silastic catheters were inserted toward the heart, tied to the vein and anchored to the surrounding tissue using 3-0 suture. Venous cannulas were externalized at the nape of the neck. All openings were closed using small wound clips. The animals were allowed a recovery period of 24 hours after surgery. The catheters were flushed with 0.3 mL of normal saline containing 500 U/mL heparin.

Nelfinavir solutions were prepared in deionized water (with 1% dimethylsulfoxide in some cases stemming from the use of concentrated stock solutions) adjusted to pH ~2.2 (DMSO) with methanesulfonic acid. Zosuquidar solutions were prepared in 5% mannitol and adjusted to pH ~2.0

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with concentrated HCl. The dosing solutions were sonicated to ensure complete dissolution and analyzed by HPLC at the end of the infusions to obtain the concentrations of solutions administered.

### **In Vivo Experimental Designs.**

*Nelfinavir only infusions.* Intravenous (jugular vein) infusions of nelfinavir (target rate of 10 mg/kg/hr) were administered to groups of either two or three rats (total of 5-8 per infusion time) for 2, 4, 6, 8 and 10 hours using a Harvard 22 syringe pump. Flow rates ranging from 1.1 to 1.8 ml/hr were based on the weight of the individual animal and theoretical dosing solution concentration. Body weights on the day of the experiment were  $274 \pm 34$  (mean  $\pm$  S.D.; n=34).

*Nelfinavir infusions with an i.v. bolus of zosuquidar.* Intravenous (jugular vein) infusions of nelfinavir (target rate of 10 mg/kg/hr) were administered to groups of three rats each for 6, 8 and 10 hours (for zosuquidar doses of 2 and 6 mg/kg) or 6 and 8 hours (for zosuquidar doses of 20 mg/kg) using a Harvard 22 syringe pump. Flow rates were adjusted for body weights, which were  $264 \pm 26$  (mean  $\pm$  S.D.; n=21) in the group of rats given zosuquidar at either 2 or 6 mg/kg and  $283 \pm 27$  (mean  $\pm$  S.D.; n=6) in the group of animals given 20 mg/kg zosuquidar. Zosuquidar (2, 6 or 20 mg/kg) was given at 4 hours as an i.v. bolus into the femoral vein.

*Tissue collection and sample preparation.* At the end of each infusion, the animals were placed under anesthesia using ketamine (1:4 ratio with saline) administered through the femoral cannula. Blood was withdrawn from the abdominal aorta into a heparinized syringe while continuing the infusion. Blood samples were centrifuged and the plasma was removed and stored at  $-20^{\circ}\text{C}$ . After the blood was drawn, the infusion was discontinued and the brain was removed from the top of the skull within 1-2 minutes. Dissected brains were rinsed with physiological saline solution to remove any external blood. They were cut into halves and frozen immediately at  $-20^{\circ}\text{C}$ .

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Plasma samples for nelfinavir and zosuquidar analyses were thawed and 2 mL of ethyl acetate:acetonitrile (90:10) and 100  $\mu$ L of 0.75 M  $\text{NH}_4\text{OH}$  were added to 100  $\mu$ L of plasma. The samples were vortexed for 4 minutes and then centrifuged for 5 minutes at room temperature and 3000 rpm. The supernatant was removed and solvent was evaporated under a stream of nitrogen. A second extraction was performed with 2 mL ethyl acetate:acetonitrile (90:10). The supernatant was added to the first dried extract and solvent was again evaporated under a nitrogen stream. The dried extracts were resuspended in mobile phase (250  $\mu$ L), pH was adjusted to  $\sim$  4 with glacial acetic acid, and the samples were filtered through a Gelman nylon acrodisc 13 mm 0.45 $\mu$ m syringe filter for HPLC analysis.

Blank and spiked control plasma samples were prepared as above with no added drug and 100  $\mu$ L of drug standard at varying concentrations in mobile phase, respectively.

Brain samples for nelfinavir and zosuquidar analysis were thawed and one half of each brain was weighed and placed in a 50-mL polyethylene conical tube. The sample was mixed with 700  $\mu$ L of 0.75 M  $\text{NH}_4\text{OH}$  and homogenized with a Tissue Tearor (BioSpec Products, Inc.) on high speed for 2 minutes. The brain homogenate was extracted with 3 mL of ethyl acetate:acetonitrile (90:10) and vortexed for 4 minutes. The samples were centrifuged for 5 minutes at room temperature and 3000 rpm. The supernatant was removed and solvent was evaporated under a stream of nitrogen. A second extraction was performed with 3 mL ethyl acetate:acetonitrile (90:10). The supernatant was added to the first dried extract and again the solvent was evaporated under a nitrogen stream. The dried extracts were resuspended in mobile phase (250  $\mu$ L), pH was adjusted to  $\sim$  4 with glacial acetic acid, and the samples were filtered through a Gelman nylon Acrodisc 13 mm 0.45 $\mu$ m syringe filter for HPLC analysis.



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Blank and spiked control brain tissue samples were prepared as above with no added drug and 100  $\mu$ L of drug standard at varying concentrations in mobile phase, respectively.

*HPLC Analyses.* Plasma and brain concentrations were determined by reversed-phase HPLC with UV detection at 254 nm (nelfinavir) and fluorescence detection at 240 nm (zosuquidar). The modular HPLC system consisted of a Waters 2690 separations module, a Waters 996 PDA detector and a Waters 474 fluorescence detector. The separations were achieved with a Supelcosil ABZ+ Plus column (5  $\mu$ m, 4.6 mm x 25 cm) at a flow rate of 0.8 mL/min. Plasma extracts were analyzed isocratically using a mobile phase consisting of 45% acetonitrile:55% ammonium acetate buffer (20 mM, pH 4.40). Brain tissue extracts were analyzed using a linear gradient from 37% acetonitrile:63% pH 7.0 ammonium acetate buffer (20 mM) to 37% acetonitrile:6.3% pH 7.0 ammonium acetate buffer (20 mM)/56.7% pH 3.0 ammonium acetate buffer (20 mM). The dosing solutions were analyzed after the infusions and actual infusion rates or doses were calculated. Tissue concentrations of nelfinavir and zosuquidar were then normalized, if necessary, to reflect the same dose per body weight for all animals.

### **Equilibrium Dialysis Experiments**

The effect of zosuquidar on the free fraction of nelfinavir in rat plasma was assessed by equilibrium dialysis using 10 mm/10 mL Spectrapor Float-A-Lyzer (Spectrum Laboratories, Inc.) dialysis membrane tubes (molecular weight cutoff = 3500). Two separate sets of pooled plasma obtained from 2-4 rats were diluted with 10 mmol/L ammonium formate buffer, pH 7.40 to a concentration of 20% plasma and spiked with a concentrated solution of nelfinavir free base in DMSO to a final concentration of  $\sim$ 10  $\mu$ g/mL. Zosuquidar in DMSO was added to half of the samples to a final concentration of  $\sim$ 3  $\mu$ g/mL. Each tube in the set was placed in a 100-mL graduated cylinder, containing a stir bar, which was filled with 10 mmol/L ammonium formate buffer, pH 7.40, to a level

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equal to the level of plasma solution within the dialysis tube. Samples were stirred continuously at room temperature. Aliquots of 1 mL were removed from the plasma side and the buffer side of the membrane at 24 and 48 hours.

Plasma and buffer samples (1 mL) were combined with 1 mL of 0.75 M  $\text{NH}_4\text{OH}$  and extracted with 2x5 mL portions of ethyl acetate:acetonitrile (90:10). Supernatants were combined, evaporated to dryness, and reconstituted in an appropriate volume of mobile phase. The sample pH was adjusted to ~ 4 with glacial acetic acid and the samples were filtered through a Gelman nylon Acrodisc 13 mm 0.20  $\mu$  syringe filter for HPLC analysis as described above.

### **Intracellular Drug Accumulation**

The influence of P-glycoprotein and breast cancer resistance protein (BCRP) over expression on cell uptake of zosuquidar was assessed in L-MDR1 cells, which over express P-glycoprotein, and Saos2 cells engineered to overexpress BCRP (Schinkel et al., 1993; Wierdl et al., 2003). The pig kidney cell line LLC-PK1 and Saos-2 cells transfected with pCDNA3 vector plasmid served as controls. Cells were seeded at 200,000/well in 6-well plates and left to attach overnight. Media was replaced with 1 ml fresh medium immediately before the addition of 10  $\mu$ l of 100X zosuquidar stock solutions in DMSO to give final concentrations of 0.25, 0.5, 0.75, 1, 5 and 10  $\mu$ M zosuquidar containing 1% DMSO. Following 20 min incubations, cells were washed three times with ice cold PBS containing 10% FBS, and 200  $\mu$ l 0.75M  $\text{NH}_4\text{OH}$  was added. Samples were rocked approximately 20 min to allow time for cell lysis, and 100  $\mu$ l of this crude extract was removed and placed in a clean glass tube for extraction following the HPLC methods outlined above. The remaining sample was stored at -20C in siliconized eppendorf tubes for protein determination. Protein assay was carried out using a BCA Protein Assay Kit (Pierce).

## Results

Linearity in the HPLC response versus drug concentration was demonstrated over the range of 0.1 – 50  $\mu\text{g/mL}$  for both nelfinavir and zosuquidar, corresponding to the approximate range of concentrations in the diluted (plasma) or concentrated (brain) HPLC samples injected. Intraday coefficients of variation (mean  $\pm$  S.D.) were 4.2%  $\pm$  1.2% and 2.7%  $\pm$  0.7% for nelfinavir and zosuquidar, respectively. Drug recoveries from spiked plasma samples were 90.2%  $\pm$  8.1% and 89.7%  $\pm$  8.7% for nelfinavir and zosuquidar, respectively, while drug recoveries from spiked brain tissue were 93.8%  $\pm$  14.6% and 94.6%  $\pm$  11.6% for nelfinavir and zosuquidar, respectively. Assays for the spiked brain and plasma samples over a range of 0.9 – 15  $\mu\text{g/mL}$  for nelfinavir and 1 – 16  $\mu\text{g/mL}$  for zosuquidar showed good linearity between the concentrations found and concentrations added ( $r^2$  values were: 0.9623 (nelfinavir, brain); 0.9871 (nelfinavir, plasma); 0.9740 (zosuquidar, brain); and 0.9935 (zosuquidar, plasma)).

Displayed in Table 1 are the mean tissue concentrations of nelfinavir and zosuquidar along with standard deviations and number of determinations at each time point and zosuquidar dose. These data were the source for Figs 1-4.

### **Nelfinavir plasma and brain tissue concentrations in the absence of P-glycoprotein inhibition.**

Fig. 1 demonstrates that steady-state plasma concentrations of nelfinavir were attained within the first 4 hours of infusion, representing 3-4 half-lives. The mean steady-state plasma concentration was 12.4  $\mu\text{g/mL}$ . From the infusion rate and mean steady-state concentration, the clearance of nelfinavir at steady-state is estimated to be 1.24 L/h/kg.

Brain concentrations of nelfinavir appeared to have attained steady-state with respect to plasma concentrations at the first sampling time though the concentrations achieved were significantly lower than the corresponding plasma concentrations (Table 1). Brain tissue concentrations were very low in

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comparison to the plasma concentrations, with an average brain:plasma concentration ratio of only  $0.06 \pm 0.03$ . Previously we estimated that the vascular content in the rat brain accounted for approximately 2% of the total brain tissue volume (Savolainen et al., 2002). Concentrations in brain parenchyma can therefore be estimated using the equation:  $C_{\text{parenchyma}} = C_{\text{br}} - V_p C_p$  where  $C_{\text{parenchyma}}$  is the parenchymal brain concentration after correction for the vascular contribution,  $C_{\text{br}}$  is the overall drug concentration in the brain,  $V_p$  is the vascular content of the brain ( $= 0.02$ ), and  $C_p$  is the drug concentration in plasma. The brain parenchyma to plasma concentration ratio for nelfinavir after this correction was  $0.037 \pm 0.027$ .

### **Zosuquidar plasma and brain tissue concentrations**

Plasma and brain tissue concentrations of zosuquidar after doses of 2 mg/kg, 6 mg/kg, and 20 mg/kg are listed in Table 1 and displayed graphically in Fig. 2 versus the nelfinavir infusion time. Zosuquidar was administered to these animals as an i.v. bolus four hours after the start of the nelfinavir infusions. While the number of time points collected was not sufficient to enable the determination of most pharmacokinetic parameters, we used the areas under the tissue concentration versus time curves between 6 and 8 hrs ( $AUC_{6-8\text{ h}}$ ) to assess the influence of zosuquidar dose on the plasma and brain concentrations because data for these time points were available at all doses. A plot of plasma  $AUC_{6-8\text{ h}}$  versus zosuquidar dose (see lower curve, Figure 4) was linear ( $r^2 = 0.9867$ ) indicating no effect of concentration of this P-glycoprotein inhibitor on its own plasma clearance.

An examination of the brain tissue concentrations versus either the dose of zosuquidar administered or plasma concentrations reveals a dramatic elevation in the brain tissue:plasma concentration ratio at the higher doses (6 mg/kg and 20 mg/kg) of zosuquidar. As shown in Table 1, brain:plasma ratios increased from  $2.8 \pm 0.3$  after a 2 mg/kg dose to  $\sim 15$  after the 6 mg/kg or 20 mg/kg doses. This abrupt increase in brain uptake of zosuquidar with an increase in dose above 2

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mg/kg is also evident in the plot (Fig. 4) of  $AUC_{6-8h}$  for the brain tissue concentrations of zosuquidar versus dose in comparison to the  $AUC_{6-8h}$  for the plasma zosuquidar concentrations versus dose.

**Effect of zosuquidar on plasma and brain concentrations of nelfinavir.**

Table 1 indicates that zosuquidar administration had no observable effect on the plasma concentrations of nelfinavir. This is also illustrated graphically in Fig. 4 by the approximately constant  $AUC_{6-8h}$  for nelfinavir plasma concentrations with increasing doses of zosuquidar.

Zosuquidar enhances the brain uptake of nelfinavir in a dose-dependent manner, as illustrated in Table 1 and Fig. 3. Brain tissue:plasma nelfinavir concentration ratios increased from  $0.06 \pm 0.03$  in the absence of zosuquidar administration and  $0.09 \pm 0.02$  between 2-6 hours after a 2 mg/kg intravenous dose of zosuquidar to  $0.85 \pm 0.19$  after 6 mg/kg, and  $1.58 \pm 0.67$  after 20 mg/kg zosuquidar. The existence of an apparent threshold concentration of zosuquidar for significant enhancement of nelfinavir uptake into brain tissue is illustrated in Fig. 4 where tissue  $AUC_{6-8h}$  values are plotted versus dose. The zosuquidar plasma concentration at this threshold appears to be ~300-400 ng/mL., though insufficient data are available to determine this value precisely.

**Effect of zosuquidar on plasma and brain concentrations of nelfinavir.**

Nelfinavir is extensively bound to plasma proteins, exhibiting a high affinity for  $\alpha$ -1 acid glycoprotein and a relatively low affinity for human serum albumin (Schon et al., 2003). Herforth et al. (Herforth et al., 2002) reported the free fraction of nelfinavir in human plasma to be 0.41%, 0.43%, and 0.41% at initial nelfinavir plasma concentrations of 1, 2, and 3  $\mu$ g/ml, respectively.

In the present study, the free fraction of nelfinavir was determined by equilibrium dialysis in two separate samples of pooled rat plasma at equilibration times of 24 and 48 h. The analyte concentrations in the diluted plasma declined from their initial values of ~10  $\mu$ g (nelfinavir)/mL and ~3  $\mu$ g (zosuquidar)/mL to ~2  $\mu$ g (nelfinavir)/mL and ~0.9  $\mu$ g (zosuquidar)/mL at 24 h and ~1.4  $\mu$ g

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(nelfinavir)/mL and  $\sim 0.5 \mu\text{g}$  (zosuquidar)/mL at 48 h due primarily to slow drug uptake into the dialysis membrane. Membrane uptake had no apparent effect on the free fraction of nelfinavir determined at 24 and 48 h. Values for the free fraction of nelfinavir in 20% plasma were  $0.055 \pm 0.006$  ((-) zosuquidar) vs.  $0.051 \pm 0.011$  ((+) zosuquidar) and  $0.021 \pm 0.006$  ((-) zosuquidar) vs.  $0.017 \pm 0.001$  ((+) zosuquidar) in the two pooled plasma samples, respectively. While the free fraction differed in the two sets of plasma, possibly due to variability in the levels of  $\alpha$ -1 acid glycoprotein, there was no discernable effect of zosuquidar on the binding of nelfinavir to plasma proteins at these concentrations. The free fraction of zosuquidar could not be quantified in one set of pooled plasma while a value of  $0.0022 \pm 0.0004$  was obtained in the other set, indicating that zosuquidar is  $\gg 99\%$  bound in 20% rat plasma and more extensively protein bound than nelfinavir. Additional studies are underway to explore the dependence of zosuquidar's plasma protein binding on its concentration in plasma.

### **Zosuquidar Intracellular Accumulation in P-glycoprotein and BCRP expressing cell lines.**

To determine if zosuquidar is transported by P-glycoprotein or BCRP, we examined its intracellular accumulation in cells over expressing P-glycoprotein and BCRP in comparison to cell lines which were not transfected. Cells were incubated for 20 minutes with increasing zosuquidar concentrations as indicated in Fig. 5. The intracellular concentration of zosuquidar was measured using HPLC and normalized to total protein content in the cell lysate. No change was observed in the intracellular levels of zosuquidar in P-glycoprotein or BCRP expressing cells suggesting that this molecule is not being effectively transported by these two efflux pumps.

## **Discussion**

The time-to-steady state results presented in Fig. 1 are consistent with the previous finding by Shetty et al. (Shetty et al., 1996), who determined that the elimination half-life for nelfinavir following

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its intravenous administration in rats at doses ranging from 25-50 mg/kg varies from 1.1-1.4 h depending on dose. The steady-state plasma concentration obtained in this study, 12.4  $\mu\text{g/mL}$ , is in good agreement with that reported in our previous study after an 8 hr infusion at the same rate (Savolainen et al., 2002), but higher than that reported by Edwards et al (Edwards et al., 2005). Also, the clearance estimate from the steady-state data in Fig. 1 is in reasonable agreement with the data of Shetty et al. (Shetty et al., 1996) who found that clearance in rats after intravenous bolus doses of nelfinavir was dose-dependent, varying from 3.61 L/h/kg at 25 mg/kg to 1.63 L/h/kg at 50 mg/kg.

Choo et al. (Choo et al., 2000) reported a brain/plasma concentration ratio of  $0.06 \pm 0.02$  in mice two hours after an i.v. injection (50 mg/kg) of nelfinavir, uncorrected for the vascular contribution, which is in good agreement with the mean of our uncorrected ratios listed in Table 1 ( $0.06 \pm 0.03$ ). Shetty et al. (Shetty et al., 1996) found a brain:plasma concentration ratio of 0.068 (uncorrected for the vascular contribution) for nelfinavir in rats four hours after an oral dose of 50 mg/kg, which also agrees well with our data. The brain parenchyma to plasma concentration ratio for nelfinavir after correcting for the vascular contribution was  $0.037 \pm 0.027$ , in reasonable agreement with the value of  $0.022 \pm 0.015$  reported previously (Savolainen et al., 2002). Thus, all studies in rodents have confirmed a limited brain uptake of nelfinavir.

Several studies have demonstrated that the P-glycoprotein efflux transporter is largely responsible for the reduced nelfinavir concentrations in brain tissue. Kim et al. (Kim et al., 1998) found that the ratio of brain concentrations of nelfinavir in *mdr1a* (-/-) mice increased by 36.3-fold relative to *mdr1a* (+/+) mice while plasma concentrations were increased only modestly (1.26-fold). Salama et al. (Salama et al., 2005) examined the disposition of nelfinavir in the brain and other tissues in P-glycoprotein competent *mdr1a1b* (+/+) mice versus P-glycoprotein double knockout *mdr1a1b* (-/-) mice. Nelfinavir concentrations in brain tissue increased 16.1-fold in double knockout mice 2 h after

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intravenous administration of nelfinavir (10 mg/kg) while plasma concentrations were unaffected by P-glycoprotein status.

Whereas zosuquidar plasma concentrations (Table 1 and Fig. 2) and AUC values (Table 1 and Fig. 4) increased approximately linearly with increasing zosuquidar dose, brain concentrations (Table 1 and Fig. 2) and brain AUC values (Table 1 and Fig. 4) exhibited more pronounced increases above a zosuquidar dose of 2 mg/kg. Thus, brain:plasma zosuquidar concentration ratios increased from  $2.8 \pm 0.3$  after a 2 mg/kg dose to  $\sim 15$  after the 6 mg/kg or 20 mg/kg doses. Dramatic increases in zosuquidar brain tissue:plasma concentration ratios at the higher doses (6 mg/kg and 20 mg/kg) of zosuquidar might be expected if zosuquidar were a substrate for P-glycoprotein as well as a P-glycoprotein inhibitor, as it would inhibit its own efflux. However, Dantzig et al. (Dantzig et al., 1999) concluded that zosuquidar is not itself a substrate for P-glycoprotein based on cell uptake/efflux data and monolayer transport data. Studies reported herein examining zosuquidar uptake/efflux in cells over expressing P-glycoprotein and BCRP in comparison to cells not expressing these transporters also suggest that zosuquidar is not effectively effluxed by P-glycoprotein or BCRP.

Studies of the binding of nelfinavir to plasma proteins in the presence and absence of zosuquidar showed no effect of zosuquidar on nelfinavir's protein binding at the concentrations employed, suggesting that the effects of zosuquidar are related to its activity as a P-glycoprotein inhibitor. However, the elevated zosuquidar brain:plasma concentration ratios with increasing zosuquidar dose could reflect changes in protein binding due to saturation with increasing dose. Preliminary evidence generated in this study suggests that zosuquidar is  $\gg 99\%$  protein bound in rat plasma.

Zosuquidar administration had no observable effect on the plasma concentrations of nelfinavir, in agreement with the findings of Choo et al. who reported no effect of zosuquidar doses up to 25



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mg/kg on plasma concentrations of nelfinavir (Choo et al., 2000). We previously demonstrated that the potent but less selective P-glycoprotein inhibitor GF120918 had no influence on plasma concentrations of nelfinavir at an intravenous dose that produced significant P-glycoprotein inhibition (Savolainen et al., 2002), results that have been recently confirmed in mice (Salama et al., 2005). Moreover, both Salama et al. (Salama et al., 2005) and Kim et al. (Kim et al., 1998) demonstrated that plasma concentrations after intravenous administration of nelfinavir or other related HIV protease inhibitors were not altered in genetic P-glycoprotein knockout mice.

The nelfinavir brain tissue:plasma concentration ratios of  $0.85 \pm 0.19$  after 6 mg/kg, and  $1.58 \pm 0.67$  after 20 mg/kg zosuquidar are comparable to those found by Choo et al. (Choo et al., 2000) though slightly lower than the nelfinavir brain:plasma ratio (1.88) attained after a single bolus dose of GF120918 (10 mg/kg) (Savolainen et al., 2002) and also lower than the ratio of 2.3 reported in *mdr1a(-/-)* knockout mice (Choo et al., 2000). Significant elevations in the nelfinavir brain:plasma ratio were realized only at zosuquidar doses above 2 mg/kg and only at doses that produced plasma concentrations of zosuquidar above 300 ng/mL, well above that necessary for >50% P-glycoprotein inhibition, which occurred at concentrations of ~50-200 ng/mL in an *ex vivo* assay by Rubin et al. (Rubin et al., 2002) The apparent threshold plasma concentration observed in our study is similar to that found by Callies et al, who determined in a study of the effect of zosuquidar on paclitaxel pharmacokinetics that maximal inhibition of P-glycoprotein in the bile canaliculi occurred at a zosuquidar  $C_{\max}$  of greater than 350 ng/mL (Callies et al., 2003). The threshold for 50% or 90% inhibition of P-glycoprotein by zosuquidar is likely to depend on the P-glycoprotein substrate being monitored.

In a Phase I trial of zosuquidar administered orally in combination with doxorubicin in cancer patients, Rubin et al. (Rubin et al., 2002) determined that the maximum-tolerated dose for oral

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zosuquidar.3HCl administered every 12 h for four days is 300 mg/m<sup>2</sup>. Cerebellar toxicity was associated with higher doses, characterized by tremors, ataxia, nystagmus, abnormal finger-to-nose testing, along with concurrent hallucinations in some patients. The plasma concentrations associated with the maximum tolerated dose on day four were 66.4-264 µg/L (C<sub>min</sub>-C<sub>max</sub>). As seen in Table 1 or Figure 2, plasma concentrations of zosuquidar in this study exceeded those attained from the maximum tolerated dose in humans at all time points obtained after the 6 mg/kg and 20 mg/kg doses while they were comparable after the 2 mg/kg dose over the time frame of the study. Rubin et al. noted that ataxia became apparent only after >24 h of dosing and was not observed when zosuquidar was given intravenously, despite the fact that similar plasma concentrations were achieved (Ford et al., 1996). This led them to suggest that a first-pass metabolite may contribute to the cerebellar toxicity. Another factor that may contribute to the cerebellar toxicity of zosuquidar is the elevated brain concentration:plasma ratio above a certain threshold plasma concentration as observed in the present study.

In conclusion, we have found that the selective P-glycoprotein inhibitor zosuquidar significantly enhances brain uptake of the HIV protease inhibitor nelfinavir when zosuquidar is administered to rats at doses > 2 mg/kg. In addition, we observed correspondingly abrupt increases in the zosuquidar brain:plasma concentration ratio in parallel with the enhancement in the nelfinavir brain:plasma concentration ratio above the threshold dose of zosuquidar. Thus, nelfinavir brain uptake appears to be linearly related to zosuquidar concentration in brain tissue. Kemper et al. concluded that the dose-limiting neurological toxicity of zosuquidar observed by Rubin et al. occurs at plasma levels of zosuquidar that are insufficient to improve the penetration of paclitaxel into brain tumors by P-glycoprotein inhibition (Kemper et al., 2004). Our results also suggest that significant enhancement in the brain uptake of nelfinavir in rats due to P-glycoprotein inhibition by zosuquidar occurs at plasma

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concentrations of zosuquidar that exceed those found by Rubin et al. in the blood of cancer patients given the maximum tolerated dose, but species-to-species differences must be taken into account in considering the implications of these results.

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## Footnotes

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## Figure Legend

Fig. 1. Concentrations of nelfinavir in plasma (■) and brain tissue (◆) (mean ± SEM) during intravenous infusions of nelfinavir at 10 mg/kg/hr.

Fig. 2. Concentrations (mean ± SEM) of zosuquidar in plasma (open symbols) and in brain tissue (closed symbols) after 2 mg/kg (Δ, ▲), 6 mg/kg (□, ■) and 20 mg/kg (◇, ◆) doses of zosuquidar during intravenous infusions of nelfinavir at 10 mg/kg/hr.

Fig. 3. Nelfinavir brain/plasma concentration ratio with and without zosuquidar (mean ± SEM) during intravenous infusions of nelfinavir at 10 mg/kg/hr.

Fig. 4. AUC<sub>6-8 hr</sub> values for nelfinavir in brain (■) and plasma (□) and zosuquidar in brain (▲) and plasma (Δ) for various doses of zosuquidar.

Fig. 5. Intracellular zosuquidar concentrations (mean ± SEM) in (A) P-glycoprotein expressing cells (L-MDR1, □) and parent cells (LLC-PK1, ■) and (B) BCRP expressing cells (○) and parent Saos2 cells (pcDNA3, ●). Cells were incubated for 20 minutes with 0.25, 0.5, 0.75, 1.0, 5.0, and 10 μM zosuquidar in the medium.

**Table 1. Nelfinavir and zosuquidar plasma concentrations, brain tissue concentrations, and brain:plasma concentration ratios during intravenous infusions of nelfinavir (10 mg/kg/hr) with or without zosuquidar administered at varying doses intravenously four hours after the start of nelfinavir infusions.**

Zosuquidar Dose (mg/kg)	Time (h) n		Nelfinavir (Mean ± SD)			Zosuquidar (Mean ± SD)		
			Plasma Conc. (µg/mL)	Brain Conc. (µg/g)	Ratio	Plasma Conc. (µg/mL)	Brain Conc. (µg/g)	Ratio
<b>0</b>	2	5	10.1 ± 4.7	0.24 ± 0.04	0.03 ± 0.01			
	4	5	14.4 ± 6.9	0.47 ± 0.20	0.04 ± 0.01			
	6	7	10.7 ± 4.7	0.78 ± 0.07	0.10 ± 0.08			
	8	8	11.6 ± 4.9	0.70 ± 0.22	0.07 ± 0.03			
	10	8	12.8 ± 9.2	0.55 ± 0.26	0.06 ± 0.02			
<b>Avg. ±SD</b>			<b>11.9 ± 1.7</b>	<b>0.55 ± .21</b>	<b>0.06 ± 0.03</b>			
<b>2</b>	6	3	16.9 ± 1.5	1.26 ± 0.30	0.07 ± 0.01	0.27 ± 0.14	0.62 ± 0.03	2.63 ± 1.14
	8	3	10.1 ± 1.3	0.80 ± 0.34	0.08 ± 0.03	0.09 ± 0.01	0.23 ± 0.01	2.58 ± 0.30
	10	3	13.2 ± 7.7	1.15 ± 0.16	0.11 ± 0.06	0.07 ± 0.02	0.21 ± 0.07	3.1 ± 1.4
	<b>Avg. ±SD</b>			<b>13.4 ± 3.4</b>	<b>1.07 ± 0.24</b>	<b>0.09 ± 0.02</b>		
					NS <sup>a</sup>			
<b>6</b>	6	3	15.3 ± 2.1	10.3 ± 3.3	0.67 ± 0.14	0.79 ± 0.10	11.0 ± 1.7	13.8 ± 0.7
	8	3	11.4 ± 3.6	12.1 ± 4.8	1.05 ± 0.11	0.76 ± 0.19	9.3 ± 0.5	12.8 ± 3.6
	10	3	18.7 ± 8.7	13.4 ± 5.1	0.83 ± 0.41	0.41 ± 0.15	6.3 ± 0.4	17.0 ± 6.0
<b>Avg. ±SD</b>			<b>15.1 ± 3.7</b>	<b>11.9 ± 1.6</b>	<b>0.85 ± 0.19</b>			<b>14.5 ± 2</b>
					*** <sup>a</sup>			*** <sup>b</sup>
<b>20</b>	6	3	16.2 ± 6.8	17.2 ± 5.5	1.10 ± 0.16	3.04 ± 0.08	37.6 ± 3.3	12.4 ± 1.3
	8	3	10.8 ± 2.2	23.1 ± 11.4	2.05 ± 0.57	1.30 ± 0.37	23.5 ± 6.8	18.4 ± 4.0
<b>Avg. ±SD</b>			<b>13.5 ± 3.8</b>	<b>20.2 ± 4.2</b>	<b>1.58 ± 0.67</b>			<b>15.4 ± 4</b>
					*** <sup>a</sup>			*** <sup>b</sup>

<sup>a</sup> Differences in nelfinavir ratios at each zosuquidar dose versus no zosuquidar were tested for significance using a oneway analysis of variance with covariate (time): NS, not significant; \*\*\*, p<0.0001

<sup>b</sup> Differences in zosuquidar ratios at the 6 mg/kg and 20 mg/kg zosuquidar doses versus the 2 mg/kg zosuquidar dose were tested for significance using a oneway analysis of variance with covariate (time): NS, not significant at p<0.05; \*\*\*, p<0.000

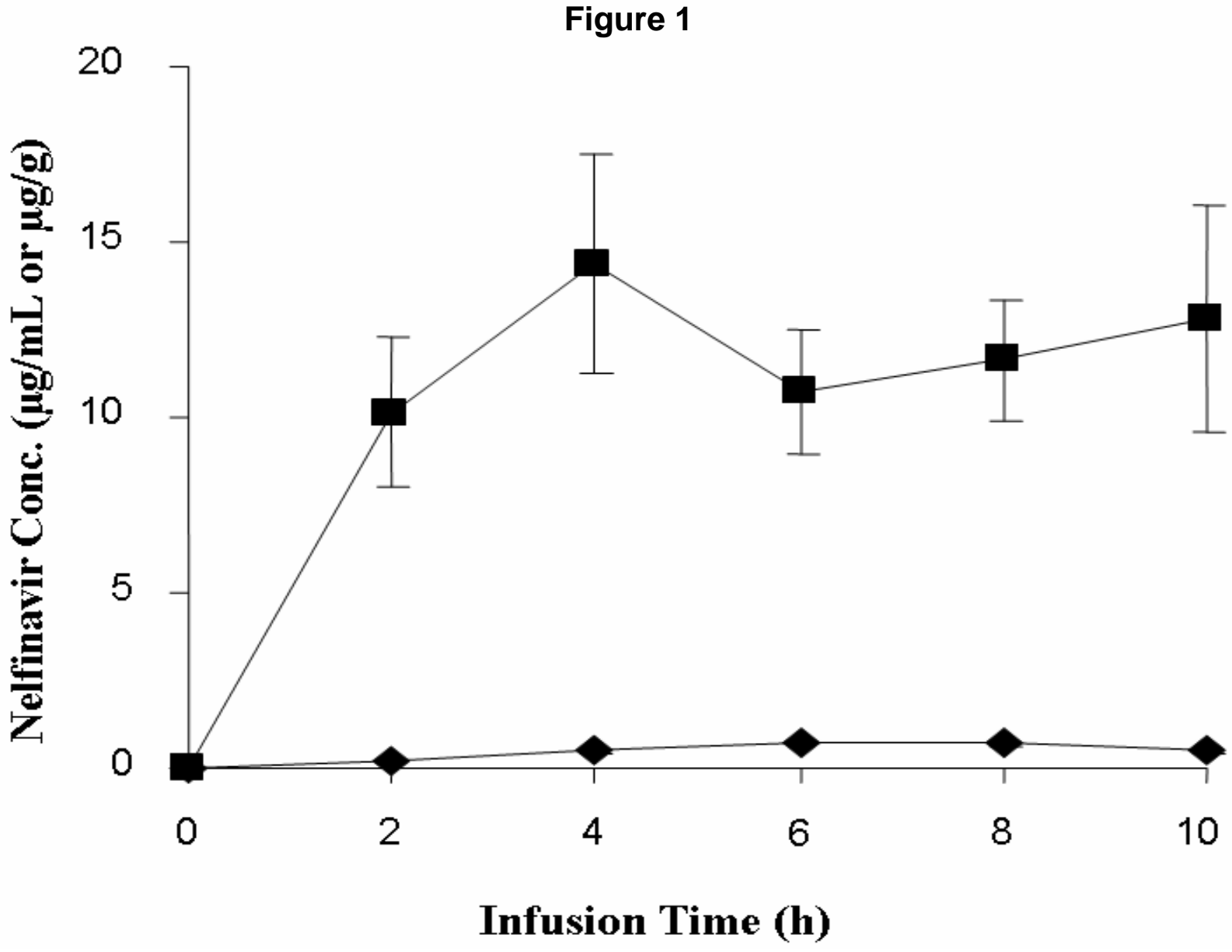


Figure 2

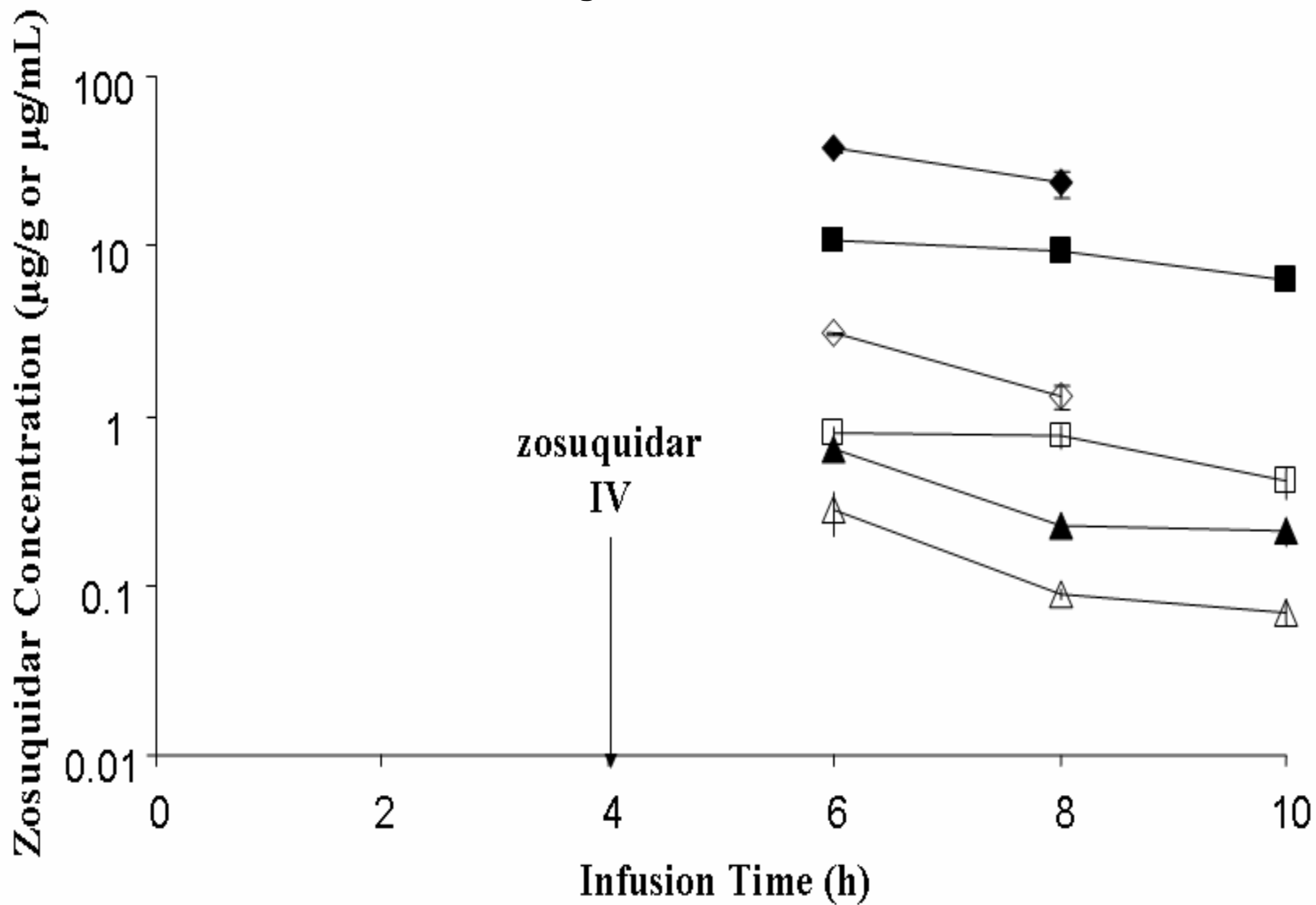
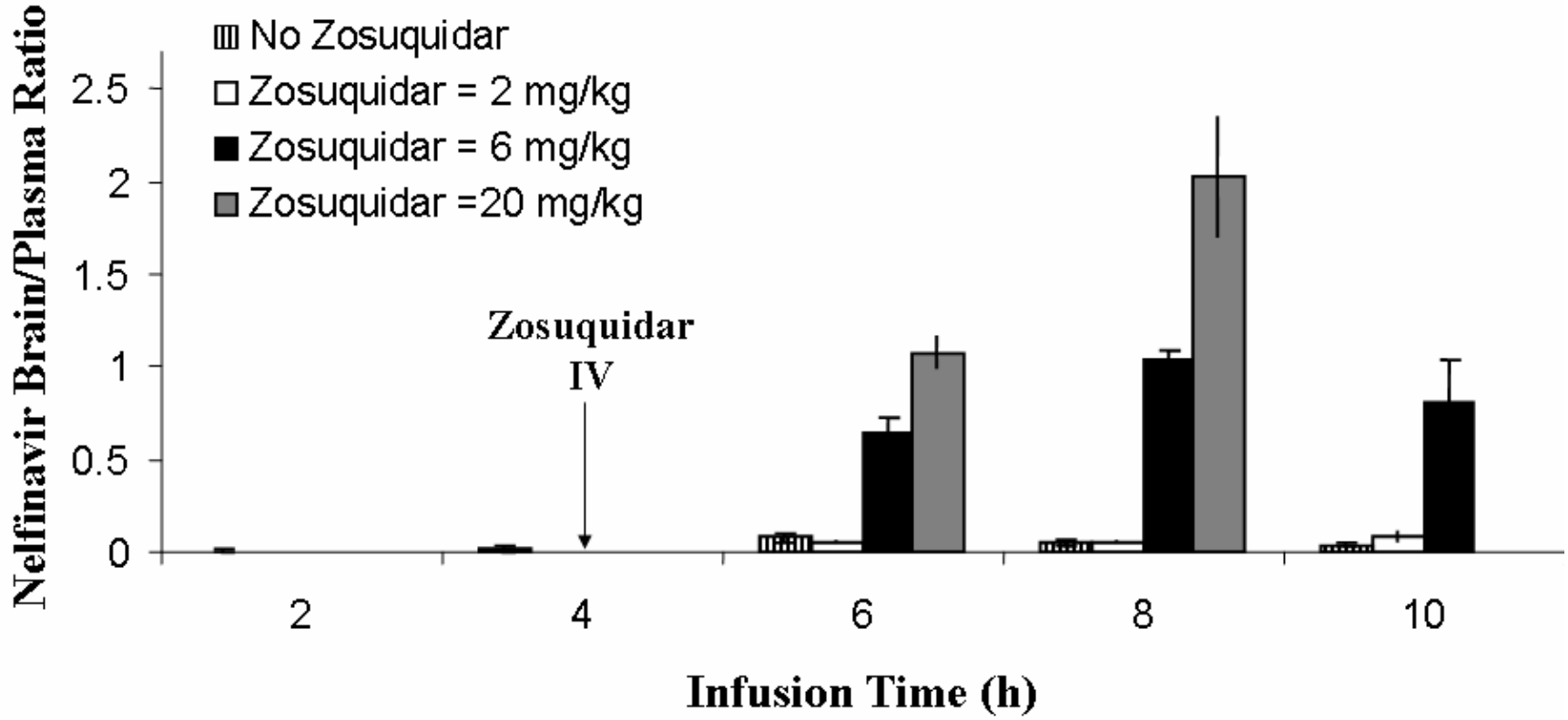
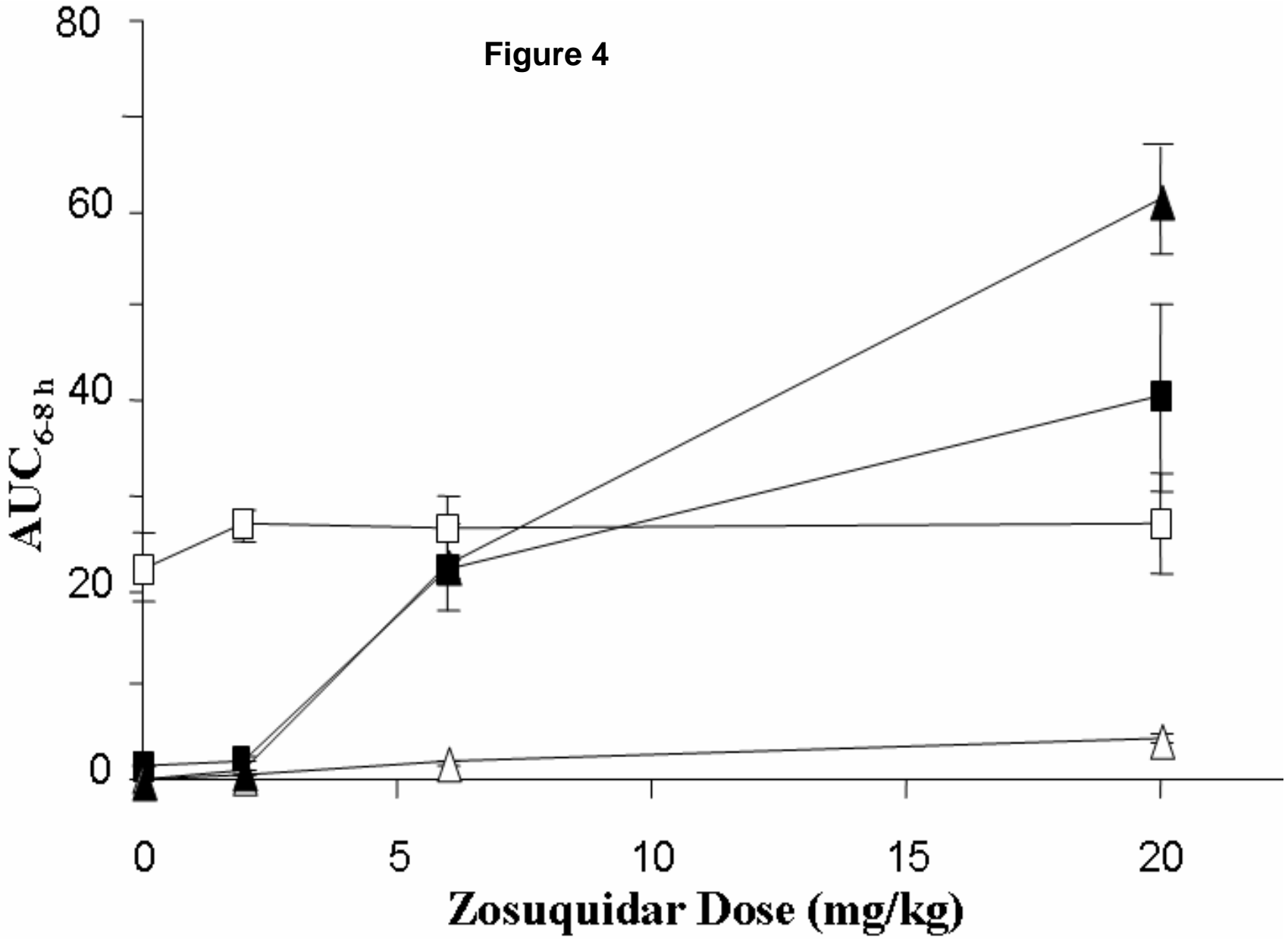


Figure 3







**Figure 5**

