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Species differences in blood-brain barrier transport of three PET radioligands with emphasis on P-glycoprotein transport

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D. Nonstandard abbreviations:

5HT receptor

serotonin receptor

CsA

Cyclosporin A

f_p

plasma protein unbound fraction

IC50

Concentration of inhibitor producing 50% of maximal inhibition

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K_p	brain-to-plasma concentration ratio
$K_{p,\text{increase}}$	increase in K_p after CsA administration
NK ₁ -receptor	Neurokinin 1 receptor
PET	Positron Emission Tomography
P-gp	P-glycoprotein
ROI	region of interest
R_{buffer}	radioactivity concentration in buffer
R_{plasma}	radioactivity concentration in plasma
SUV	standardized uptake value
SUV_{increase}	increase in SUV after CsA administration

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Abstract

Species differences occur in the brain concentrations of drugs, but the reasons for these differences are not yet apparent. This study was designed to compare brain uptake of three radiolabelled P-glycoprotein (P-gp) substrates across species using Positron Emission Tomography (PET). Brain concentrations and brain-to-plasma ratios were compared; [^{11}C]verapamil in rats, guinea pigs and monkeys, [^{11}C]GR205171 in rats, guinea pigs, monkeys and humans, and [^{18}F]altanserin in rats, minipigs and humans. The fraction of the unbound radioligand in plasma was studied along with its metabolism. The effect of P-gp inhibition was investigated by administering cyclosporin A (CsA). Pronounced species differences were found in the brain and brain-to-plasma concentrations of [^{11}C]verapamil, [^{11}C]GR205171 and [^{18}F]altanserin with higher brain distribution in humans, monkeys and minipigs than in rats and guinea pigs. For example, the brain-to-plasma ratio of [^{11}C]GR205171 was almost 9-fold higher in humans compared to rats. The species differences were still present after P-gp inhibition although the increase in brain concentrations after P-gp inhibition was somewhat greater in rats than in the other species. Differences in plasma protein binding and metabolism did not explain the species-related differences. The findings are important for interpretation of brain drug delivery when extrapolating preclinical data to humans. Compounds found to be P-gp substrates in rodents are likely to also be substrates in higher species but sufficient blood-brain barrier permeability may be retained in humans to allow the compound to act at intracerebral targets.

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Introduction

Species differences in receptor and enzyme systems are well known, but very little is known about species differences in the transport of drug molecules across the blood-brain barrier. Transport across this barrier occurs by passive diffusion either alone or in combination with active influx or efflux. P-glycoprotein (P-gp; ABCB1; MDR1) is an ATP-dependent efflux transporter that carries a wide range of drug molecules at the blood-brain barrier as well as in other organs such as the liver, kidney and gastrointestinal tract. It has a profound influence on brain concentrations of its substrates. Due to its non-invasiveness, Positron Emission Tomography (PET) is one of the few methods that allows for direct *in vivo* comparisons between preclinical species and humans regarding drug interactions with P-gp.

[¹¹C]verapamil is the most frequently used PET radioligand for P-gp studies (Hendrikse et al., 1999; Bart et al., 2003; Sasongko et al., 2005; Lee et al., 2006; Syvänen et al., 2006). Other examples include [¹¹C]carazolol, [¹⁸F]fluorocarazolol, [¹¹C]carvedilol, [¹¹C]GR218231, [¹¹C]loperamide and [¹¹C]desmethyl loperamide (Doze et al., 2000; Bart et al., 2005; de Vries et al., 2005; Lazarova et al., 2008; Zoghbi et al., 2008). Some radioligands shown to be P-gp substrates in rodents are nonetheless taken up by the brain in primates, indicating that there may be species differences in P-gp function. For example, Liow *et al.* have shown that the 5HT_{1A} receptor antagonist [¹¹C]RWAY is a P-gp substrate in the rat and mouse and displays a low brain uptake in these species (Liow et al., 2007). However, uptake of [¹¹C]RWAY in human and monkey brains is relatively high (Yasuno et al., 2006; Zhang et al., 2007). Similarly, while uptake of the chemically

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related 5HT_{1A} receptor antagonist [¹⁸F]MPPF by the human brain is relatively high, the molecule has been shown to be a P-gp substrate in rats (Passchier et al., 2000a; Passchier et al., 2000b). Further, GR205171, an NK₁ receptor antagonist, is a P-gp substrate in rodents (Rupniak et al., 2003) but uptake of radioactively labelled [¹¹C]GR205171 is high in human and monkey brains (Bergström et al., 2000; Furmark et al., 2005; Michelgard et al., 2007). Uptake of [¹⁸F]altanserin, a 5HT_{2A} receptor antagonist, in human, baboon and pig brains is also relatively high (Biver et al., 1994; Staley et al., 2001). However, initial *ex vivo* studies of [¹⁸F]altanserin in rats have shown relatively low brain concentrations (unpublished data, NRU Copenhagen, Denmark).

One explanation for the species differences in brain concentrations observed with these radioligands could be the existence of a species difference in P-gp function, despite the high degree of homology in the amino acid content of P-gp in different species (Table 1). Several *in vitro* studies have shown that the transport capacity of P-gp substrates differs among cell lines transfected with P-gp from different species, indicating a species difference in substrate recognition (Yamazaki et al., 2001; Ohe et al., 2003; Katoh et al., 2006; Xia et al., 2006; Baltes et al., 2007). Besides differences in substrate affinity, differences in P-gp expression could also result in differences in P-gp transport capacity. *In vivo* evidence for species differences in P-gp function has so far only been reported by one group, who compared rats and guinea pigs and found that higher concentrations of the P-gp inhibitor GF-120918 were needed in guinea pigs than in rats to achieve the same increase in brain concentrations of the P-gp substrate SB-487946 (Cutler et al., 2006). In contrast, when Hsiao *et al.* compared the increase of [¹¹C]verapamil in the human and rat

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brain after treatment with low doses of the potent *in vivo* P-gp inhibitor cyclosporin A (CsA) they did not observe any species differences (Hsiao et al., 2006). Other explanations could be species differences in blood-brain barrier transport that are not related to P-gp function, but rather to other causes, e.g. tightness of the endothelial cell tight junctions or expression of other active efflux transporters. Differences in intrabrain distribution due to differences in lipophilic content of brain tissue, and in the degree of plasma protein binding, metabolism and elimination from plasma could also lead to different levels of brain uptake among species.

The *in vivo* brain uptake of [^{11}C]verapamil, [^{11}C]GR205171 and of [^{18}F]altanserin in several species was investigated in this paper. Interspecies comparisons of brain concentrations and brain-to-plasma concentration ratios, fraction of unbound radioligand in plasma and radioligand metabolism were carried out. The effect of P-gp was studied by administering CsA to a subgroup of animals.

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Materials and Methods

General

The radioligands [¹¹C]verapamil, [¹¹C]GR205171 and [¹⁸F]altanserin, Figure 1, were synthesised as previously reported (Lemaire et al., 1991; Bergström et al., 2000; Syvänen et al., 2006). All chemicals were obtained commercially and were of reagent grade or better. The P-gp inhibitor CsA (Sandimmun®, Novartis, Basel, Switzerland) was diluted to the desired concentration in saline. All animal experiments were approved by the Uppsala Animal Ethics Committee (C117/4 and C 153/4) and the Danish Animal Research Inspectorate (Journal number 2003/561-745). The clinical data used in this paper were acquired from clinical studies in healthy volunteers (Syvänen, 2003; Liptrot et al., 2004) and were performed in accordance with the Helsinki declaration. A summary of the number of subjects for each radioligand and species is given in Table 2.

Anaesthesia and surgical procedures

Before the experiments, rats and guinea pigs were housed in ventilated cabinets at a room temperature of 20±2°C, were maintained on a 12 h light: 12 h dark cycle and were given unlimited access to food and water. Gaseous anaesthesia with isoflurane 2.6% (Baxter Medical AB, Kista, Sweden) in a mixture of oxygen and medical air (40%/60%, AGA Gas AB, Sundbyberg, Sweden) was used for the rat and guinea pig studies with [¹¹C]GR205171 and [¹¹C]verapamil, while hyponorm/midazolam (VetaPharma Ltd., Leeds, United Kingdom/Hameln Pharmaceuticals, Hameln, Germany) was used for the studies with [¹⁸F]altanserin. After anaesthesia, a femoral artery cannula was inserted for blood sampling and an intravenous catheter was inserted into the left femoral vein in

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guinea pigs and the tail vein in rats for radioligand and CsA administration. The [^{11}C]GR205171 and [^{11}C]verapamil uptake in rats and guinea pigs was studied dynamically with PET, while the brain uptake of [^{18}F]altanserin was measured after euthanization of the animals (*ex vivo* studies).

The minipigs used for the [^{18}F]altanserin studies were initially anaesthetized with 0.15 ml/kg i.m. of midazolam (Hameln Pharmaceuticals, Hameln, Germany) plus Zoletil 50 vet. (Virbac Animal Health, Carros, France). One ampoule of Zoletil 50 vet (containing 125 mg each of zolazepam and tiletamine) was dissolved in 8 ml midazolam solution (1 mg/ml). The anaesthesia was maintained with i.v. propofol (1 ml/kg/h) (Fresenius Kabi AB, Uppsala, Sweden). The minipigs were intubated endotracheally and ventilated during the entire experiment. During the scan, they were placed on a heating carpet and covered to maintain body temperature. Catheters were surgically inserted in the femoral vein for radioligand and CsA administration, and into the femoral artery for blood sampling. Body temperature and physiological functions (blood pressure, blood oxygenation and heart rate) were monitored continuously and haematocrit, blood glucose and acid–base parameters (pH, pCO₂, pO₂, H₂CO₃ and O₂ saturation) were measured in full blood samples on an ABL system (625, Radiometer, Copenhagen, Denmark) at regular intervals. Deviations from normal values were corrected appropriately.

The cynomolgus monkeys were transported to the investigation site at Uppsala Imanet on the morning of the experiment. Venous catheters were inserted in both hind legs and propofol (50 mg, Lipuro®, B.Brown Medical AB, Bromma, Sweden) was administered

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to induce anaesthesia. The catheters were also used for administration of the radioligands and Ringer-Acetate (2 ml/kg/h, Fresenius Kabi AB, Uppsala, Sweden). The animals were kept anaesthetised with 1.3 - 2.5% sevoflurane (Abbott Scandinavia AB, Solna, Sweden) via tracheal intubation during the PET scan. A venous catheter in the tail was used for blood sampling. One monkey had two PET scans and another had three. The PET scan was started when the radioligand was administered. Data from an earlier study with [¹¹C]GR205171 in rhesus monkeys were also included in this study (Sylvänen et al., 2007).

Blood (0.2 ml) was sampled 0.5, 1, 3, 5, 10, 15, 20, 30, 45 and 60 min after administration of all compounds for all species and the radioactivity concentration in blood and plasma was measured in a well counter that was cross-calibrated with the PET system. Additional blood samples (0.3 ml) were obtained at 0, 15, 30 and 60 min for measurement of CsA concentrations in whole blood using an immunoenzymatic assay (CEDIA, Microgenics, Ekerö, Sweden) with a limit of detection of 25 ng/mL. Three to 5 blood samples (1-2 ml) were obtained for metabolite analysis in minipigs, monkeys and humans during the scan.

PET scanners

In rodents, a microPET R4 scanner (Concorde Microsystems, Knoxville, TN, USA) was used for [¹¹C]GR205171 and [¹¹C]verapamil studies. In minipigs, a combined PET/CT Discovery LS Scanner (General Electrics, Milwaukee, WI, USA) was used for [¹⁸F]altanserin studies and, in monkeys, a Hamamatsu SHR-7700 tomograph

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(Hamamatsu Photonics, Hamamatsu, Japan) was used for [^{11}C]GR205171 and [^{11}C]verapamil studies. The human [^{11}C]GR205171 studies were performed using an ECAT EXACT HR+ scanner (Siemens/CTI, Knoxville, TN, USA) and the [^{18}F]altanserin studies used a GE Advance scanner (General Electrics, Milwaukee, WI, USA).

Transmission scans, used for correction of attenuation, were run prior to the emission scans.

Studies with [^{11}C]verapamil

Six rats (mean weight 412 ± 45 g) received [^{11}C]verapamil as a bolus injection of 26 ± 11 MBq. Three of them received a bolus dose of 22.5 mg/kg CsA followed by a constant infusion of 7.5 mg/kg/h, starting 20-25 min prior to administration of the radioligand. The same protocol was used for six guinea pigs (mean weight 437 ± 48 g) at a [^{11}C]verapamil dose of 15 ± 5 MBq. One female cynomolgus monkey (weight 3.8 kg) received a bolus injection of [^{11}C]verapamil 68 MBq. Four and half hours later, a 15 mg/kg CsA bolus was followed by a constant infusion of 5 mg/kg/h, started 20-25 min prior to the second [^{11}C]verapamil bolus of 74 MBq. Regions of interest (ROIs) were drawn over the transaxial brain images in the region of the cerebellum.

Studies with [^{11}C]GR205171

Nine rats (mean weight 400 ± 26 g) received a bolus injection of 26 ± 13 MBq [^{11}C]GR205171. Three of them received a bolus dose of 22.5 mg/kg CsA followed by a constant infusion of 7.5 mg/kg/h, while three others were administered a bolus dose of 7.5 mg/kg CsA followed by a constant infusion of 2.5 mg/kg/h, starting 20-25 min prior

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to administration of the radioligand. Six guinea pigs (mean weight 488 ± 58 g) received a bolus injection of 27 ± 8 MBq [^{11}C]GR205171. Three of them received a bolus dose of 22.5 mg/kg CsA followed by a constant infusion of 7.5 mg/kg/h, starting 20-25 min prior to the radioligand administration. The first of two female cynomolgus monkeys (weights 6.4 and 3.8 kg) received [^{11}C]GR205171 as a single bolus of 95 MBq, and 4.5 hours later received a bolus dose of 15 mg/kg CsA and a constant infusion of 5 mg/kg, 20-25 min prior to a second bolus dose of 112 MBq [^{11}C]GR205171. The second animal received one single bolus dose of 78 MBq [^{11}C]GR205171. ROIs were drawn over the transaxial brain in the region of the cerebellum where the distribution of NK₁ receptors is negligible (Bergström et al., 2000; Haneda et al., 2007). In the monkeys, ROIs were also drawn over the striatum where NK₁ receptors are abundant.

Studies with [^{18}F]altanserin

Nine rats (mean weight 371 ± 70 g) received 8 ± 4 MBq [^{18}F]altanserin as a bolus injection. Four of them received a 22.5 mg/kg bolus of CsA followed by a constant infusion of 7.5 mg/kg/h, starting 20-25 min prior to the administration of radioligand. Six Göttingen minipigs (mean weight 31 ± 8 kg) received a bolus injection of 197 ± 154 MBq [^{18}F]altanserin. One of them received a bolus of 22.5 mg/kg of CsA followed by a constant infusion of 7.5 mg/kg/h starting 20-25 min prior to the radioligand administration. In rat studies, the brain was dissected at 60 min and the radioactivity was measured in the cerebellum, where the distribution of 5HT_{2A} receptors is negligible, and frontal cortex, in which the density of 5HT_{2A} receptors is high (Biver et al., 1994). In

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minipig studies, ROIs were automatically applied in the region of the cerebellum and the frontal cortex using a minipig brain atlas (Watanabe et al., 2001).

Plasma protein binding

The extent of plasma protein binding was determined using equilibrium dialysis. Plasma samples were obtained, 1-2 MBq of the radioligand was added to each sample, and 500 μ L of plasma was then transferred to a dialysis chamber with a semipermeable membrane separating the other chamber containing 500 μ L of PBS buffer. After the chambers had been shaken in a 37°C water bath for 2 hours, a sample from each chamber was removed and the radioactivity concentrations were measured. The plasma protein unbound fraction, f_p , was calculated as:

$$f_p = \frac{R_{buffer}}{R_{plasma}} * 100 \quad (1)$$

R_{buffer} and R_{plasma} are the radioactivity concentrations in the buffer and plasma chambers, respectively. All values are based on $n \geq 3$ unless otherwise stated, and are reported as means and standard deviations. A two-tailed t-test was used to test whether the difference in plasma protein binding between plasma containing and not containing CsA was statistically significant.

Metabolism

The metabolism of [11 C]verapamil and [11 C]GR205171 was evaluated in rat and guinea pig plasma. The metabolism of [18 F]altanserin in rats has been studied previously (Tan et

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al., 1999). The metabolism of [¹¹C]verapamil and [¹¹C]GR205171 in monkey plasma and the metabolism of [¹⁸F]altanserin in minipigs was performed in parallel with the imaging studies. The methods for separating and analysing parent and metabolite fractions of [¹¹C]verapamil, [¹¹C]GR205171 and [¹⁸F]altanserin have been published previously (Bergström et al., 2000; Pinborg et al., 2003; Syvänen et al., 2006).

Data analysis

Radioactivity measurements in plasma were corrected for metabolism before analysis. The time-activity profiles in the brain and plasma were expressed using the Standardized Uptake Values (SUV; normalised concentrations) to enable comparison between scans.

$$SUV = \frac{\text{Measured Radioactivity in tissue}}{\text{Injected Radioactivity} / \text{Body Weight}} \quad (2)$$

To account for individual and species differences in the plasma concentrations, which are the driving force for the brain concentrations, the brain-to-plasma ratio K_p was calculated using the SUV in plasma and brain.

$$K_p = \frac{SUV \text{ brain region}}{SUV \text{ plasma}} \quad (3)$$

SUV in brain and plasma and K_p were calculated over the scanning time, one value for each PET time frame. Average SUV and K_p values for each animal were also determined from calculations of SUV and K_p every 5 min between 0 and 60 min. All values are

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reported as means and standard deviations. The effect of P-gp inhibition was calculated by comparing SUV or K_p in CsA-untreated and -treated animals.

$$SUV_{increase} = \frac{SUV_{average\ 0-60\ min\ with\ CsA}}{SUV_{average\ 0-60\ min\ baseline}} \quad (4)$$

$$K_{p, increase} = \frac{K_{p\ average\ 0-60\ min\ with\ CsA}}{K_{p\ average\ 0-60\ min\ baseline}} \quad (5)$$

$SUV_{increase}$ and $K_{p, increase}$ were calculated from the average values for SUV and K_p .

$K_{p, increase}$ takes into account CsA-induced differences in the plasma pharmacokinetics and may therefore be a more reliable parameter than $SUV_{increase}$ for assessing the change in CsA-induced P-gp inhibition. A one-tailed t-test was used to see if the increase was statistically significant.

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Results

Species differences in radioligand brain and plasma concentrations

Distribution to the brain of the three studied radioligands [^{11}C]verapamil, [^{11}C]GR205171 and [^{18}F]altanserin were clearly higher in humans, monkeys and minipigs than in rats and guinea pigs, as described with brain concentrations, SUV, and the brain-to-plasma concentration ratios, K_p (Figures 2 – 4, Table 3). For [^{11}C]verapamil, the brain distribution was highest in monkeys, followed by rats and guinea pigs with K_p -values of 4.6 to 1.1 and 0.65, respectively (Table 3). Monkeys had a 4-fold higher brain distribution ratio (K_p) than rats. The same trend was observed with [^{11}C]GR205171 and [^{18}F]altanserin, where monkeys and minipigs showed higher brain uptake than rodents (Table 3). PET images with [^{11}C]GR205171 in monkey, guinea pig and rat are shown in Figure 5. Humans had a [^{11}C]GR205171 K_p ratio of 30, while rats had a ratio of 3.4, thus an 8.6-fold higher brain distribution in humans than in rats, and 3-fold higher in humans than in monkeys. The K_p of [^{18}F]altanserin was 4.5-fold higher in humans than in rats.

The metabolite corrected plasma SUV values after [^{11}C]verapamil administration, describing the drug concentrations in plasma in relation to dose administered (Equation 2), were highest in guinea pigs, followed by rats and monkeys. For [^{11}C]GR205171 plasma SUVs were highest in monkeys, followed by guinea pigs and then humans and rats. Plasma SUV values of [^{18}F]altanserin were highest in rats, followed by humans and minipigs. Thus, in contrast to the brain concentrations of the three radioligands, no tendency towards lower concentrations in the rodents than in the higher species was observed.

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Effect of CsA induced P-gp inhibition

The potent P-gp inhibitor CsA was administered to a subgroup of the animals (not to humans). Since plasma concentrations are the driving force for brain concentrations, it is more relevant to compare the K_p than the SUV values with and without CsA treatment to obtain information on how much of the increase is due to P-gp inhibition. K_p was increased in all species after CsA administration, apart from the K_p of [^{18}F]altanserin in the minipig (Table 4). The increase was highest in rats for all three radioligands. From a clinical point of view, where the actual brain concentrations are of interest for a given dose, the increase in SUV may also be of interest. Both, $K_{p,\text{increase}}$ and $\text{SUV}_{\text{increase}}$ are therefore shown in Table 4. PET images with and without CsA treatment for [^{11}C]GR205171 in monkey, guinea pig and rat are shown in Figure 5, for [^{11}C]verapamil in the monkey are shown in Figure 6 and [^{18}F]altanserin in the mini pig are shown in Figure 7.

The CsA concentrations influence the degree of P-gp inhibition. The mean CsA concentrations were $19.1 \pm 9.3 \mu\text{g/mL}$ in guinea pigs and $11.7 \pm 1.7 \mu\text{g/mL}$ in rats. Three of the [^{11}C]GR205171 administered rats received a lower dose of CsA which resulted in a mean CsA concentration of $2.2 \pm 0.5 \mu\text{g/mL}$. The two monkeys treated with CsA had somewhat different CsA concentrations despite being dosed according to weight. The monkey receiving [^{11}C]verapamil showed CsA concentrations ranging from $10 \mu\text{g/mL}$ at the beginning of the scan to $6.6 \mu\text{g/mL}$ at the end, while the monkey receiving [^{11}C]GR205171 showed CsA concentrations ranging from $20 \mu\text{g/mL}$ at the beginning of

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the scan to 16 $\mu\text{g/mL}$ at the end. In the [^{18}F]altanserin administered minipig the CsA concentration continued to increase throughout the scan, ranging from 62 $\mu\text{g/mL}$ at the start to 73 $\mu\text{g/mL}$ at 60 min, i.e. it was about three times higher than in rats. In spite of this, the minipig studied did not show an increase in K_p , although the brain SUV increased almost 2-fold (Table 4).

Metabolism of the radioligands

The metabolism of [^{11}C]verapamil in plasma was affected by CsA, which resulted in a lower fraction of intact [^{11}C]verapamil in CsA-treated animals. In the monkey; half of the radioactivity originated from metabolites at 24 min in CsA-untreated animal, compared to 17 min in the CsA-treated animal. CsA did not affect the metabolism of [^{11}C]GR205171 and [^{18}F]altanserin. The metabolism of [^{11}C]GR205171 was very similar in rats and guinea pigs. After about 40 min, half of the radioactivity in plasma from these species originated from metabolites whereas, in humans and monkeys, this point had been reached by 20 min, thus a slower metabolism in the rodents. Plasma samples from rats 4 hours after [^{18}F]altanserin administration consisted of around 80% intact radioligand (Tan et al., 1999). In [^{18}F]altanserin administered minipigs and humans, half of the radioactivity in plasma originated from metabolites at around 80 and 90 min, respectively.

The plasma protein unbound fraction

The f_p values for radioligands in the different species are shown in Table 5. The f_p for [^{11}C]verapamil was highest in monkeys, followed by rats and guinea pigs. No species

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difference in protein binding was observed for [^{11}C]GR205171 and [^{18}F]altanserin, although the f_p was very low for [^{18}F]altanserin making a reliable measurement difficult. A general trend was that the f_p was somewhat increased after CsA administration, but the increase was only significant in [^{11}C]GR205171 administered rats.

Discussion

This paper reports pronounced species differences in the brain pharmacokinetics of three PET radioligands that are P-gp substrates, both with and without CsA-induced P-gp inhibition. Rats and guinea pigs showed lower K_p ratios for the radioligands studied, than minipigs, monkeys and humans. This could be due to more P-gp present in the blood-brain barrier of rodents, or due to more efficient P-gp in these species leading to a higher P-gp mediated transport capacity. Alternatively, there could be species related differences in plasma protein binding leading to different concentrations of molecules available for transport across the blood-brain barrier. Finally, also differences in the intrabrain distribution due to differences in brain tissue properties could lead to the observed species differences.

If transport capacity would be the only explanation to the differences observed when P-gp is functioning normally, complete P-gp inhibition should result in similar brain-to-blood concentration ratios (K_p) across all species. However, the large species differences in brain concentrations were not abolished after P-gp inhibition in the present paper.

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Hence, our conclusion is that the differences in P-gp transport capacity alone do not explain the observed species differences.

In vitro studies have demonstrated differences in the efficacy of P-gp-mediated transport in cell lines transfected with the MDR1 gene (gene coding for P-gp) from different species. In general, most of the evaluated substrates are transported to a higher extent by mouse or rat P-gp than by human P-gp (Yamazaki et al., 2001; Ohe et al., 2003; Katoh et al., 2006; Baltes et al., 2007). However, there are exceptions. CsA, for example, was more effectively transported in cells transfected with human P-gp than in those transfected with rat or mouse P-gp (Katoh et al., 2006; Baltes et al., 2007). Another *in vitro* study showed that the IC₅₀ of typical P-gp inhibitors differed with species and substrate (Suzuyama et al., 2007). Cutler *et al.* demonstrated *in vivo* differences between rats and guinea pigs in the IC₉₀ of the P-gp inhibitor GF-120918 (Cutler et al., 2006). They showed that a higher plasma concentration of GF-120918 was needed in guinea pigs than in rats to achieve the same increase in brain concentrations of a P-gp substrate, SB-487946. Taken together, the *in vitro* and *in vivo* studies indicate that both transport capacity of P-gp and the IC₅₀ values of P-gp inhibitors may differ among species. This would support the hypothesis that there are species differences in both P-gp transport and the IC₅₀ of CsA, and may explain the observed *in vivo* species differences at both baseline and during P-gp inhibition in the present paper.

Species differences in intrabrain distribution could also be a relevant explanation for our results. The total substrate brain concentrations observed with PET will depend on the

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binding potential of the substrate to brain tissue. In this instance, the term binding is taken to include both specific binding to receptors and nonspecific binding to brain tissue components. Brain concentrations of the radioligand will be higher in species with a higher binding potential to brain tissue than in those with a lower binding potential. Species differences in passive transport over the blood-brain barrier would lead to the same outcome as differences in the intrabrain distribution since both active and passive transport contribute to the K_p . However, it is unlikely that the physico-chemical properties of, and tightness of tight junctions in, the blood-brain barrier itself would differ to a large extent among species (Cserr and Bundgaard, 1984). Further, the expression of other active transporters such as Mrp, Oat, and Oatp could also contribute to the species differences noted.

Other possible explanations could be differences in the plasma protein binding of the substrate. A lower level of plasma protein binding in one species would, given the same total plasma concentration, contribute to higher brain concentrations, as more substrate molecules would be available for transport across the blood-brain barrier. Indeed, in our study, plasma protein binding was somewhat lower in the higher species than in rats and guinea pigs, but the differences were not large enough to explain the species differences in brain concentrations.

To account for species-related differences in the metabolism of the radioligands, which could have contributed to differences in brain concentrations, the plasma concentrations were corrected for metabolites carrying the radioactive label in the calculation of K_p in

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our study. Nonetheless, the entry of radioactive metabolites into the brain could also conceivably have affected our results, since the brain kinetics could vary among species depending on the relative fractions of intact radioligand and radiolabelled metabolites. This is not, however, a very likely explanation for the species differences seen in this study since the differences appeared right from the start of the PET scans, when most of the radioactivity originated from the intact radioligand. Previous studies focusing in more detail on the metabolism of [^{11}C]verapamil and [^{18}F]altanserin in the rat has showed that after [^{11}C]verapamil i.v. administration most activity in the brain originates from intact [^{11}C]verapamil and that no lipophilic metabolites are formed during the metabolism of [^{18}F]altanserin (Tan et al., 1999; Luurtsema et al., 2005; Syvänen et al., 2008). Thus, metabolism and plasma protein binding were ruled out as explanations for the species differences.

There were some differences in the anesthesia protocols of the different experiments. Hypnorm, which was used in the [^{18}F]altanserin study in rat, contains fentanyl. Fentanyl has been shown to be a P-gp substrate (Henthorn et al., 1999) and could therefore in theory increase the uptake of [^{18}F]altanserin by competing for P-gp substrate binding sites. Isoflurane, which was used in the rodent [^{11}C]verapamil and [^{11}C]GR205171 studies, has been shown to moderately increase radioligand binding (Tsukada et al., 1999; Elfving et al., 2003; Harada et al., 2004). Both Hypnorm and isoflurane anesthesia may therefore reduce the measured difference in uptake between rodents and humans rather than to be the cause of the difference. Isoflurane has been shown to decrease the cerebral blood flow (Tsukada et al., 1999). This could indirectly change the radioligand uptake in

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brain and may be a confounding factor for the results. However, based on the results obtain by Tsukada *et al.* (Tsukada et al., 1999), the change in cerebral blood flow was too small to explain the differences between the rats and the humans.

Although our study of [¹¹C]verapamil included only two PET-scans in monkeys, one untreated and one receiving CsA, our results are in line with those in other publications. For example, Lee *et al.* studied uptake of [¹¹C]verapamil after P-gp inhibition, and reported that a 2.3-fold increase in K_p values after a 20 mg/kg infusion over 2 hours of the P-gp inhibitor PSC833 (P-gp inhibition potency is similar to that of CsA), starting one hour before the PET scan (Lee et al., 2006). This is to be compared with our 3.6-fold increase.

The CsA concentrations in our study were rather similar between species, apart from in the minipig. The guinea pigs had higher CsA concentrations than the rats. Still, the increase in K_p was smaller in the guinea pigs than in the rats. This may underestimate the K_p difference between guinea pigs and rats, rather than being an explanation to the difference. The CsA-treated [¹¹C]verapamil monkey had lower CsA plasma concentrations than the corresponding rodents, which may have resulted in relative underestimation of $K_{p, \text{increase}}$ in the monkey.

Liow *et al.* speculated that P-gp inhibition caused a smaller increase in [¹¹C]RWAY concentrations in the cerebellum than in 5HT_{1A} receptor-rich regions (Liow et al., 2007). Our results showed a similar trend; SUV_{increase} and $K_{p, \text{increase}}$ values were somewhat

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smaller in the cerebellum than in the high binding regions of striatum and frontal cortex for [^{11}C]GR205171 and [^{18}F]altanserin, respectively. In line with these results, Lacán *et al.* showed in a recent paper, a somewhat larger increase in brain uptake of [^{18}F]MPPF in the $5\text{HT}_{1\text{A}}$ receptor rich area of hippocampus compared to cerebellum after P-gp inhibition (Lacan et al., 2008). Regional differences could be attributed to heterogeneous distribution of P-gp within the brain. In the study by Lacán *et al.* P-gp protein was expressed more in cerebellum than in other brain regions, a somewhat contradictory finding to the results reported above (Lacan et al., 2008).

In the development of new drugs, *in vitro* methods of screening for P-gp-mediated transport and *in vivo* animal models are usually utilized before clinical experiments in humans. If a drug that is intended for a target inside the brain is found to be a P-gp substrate or is found in a rat model to have low brain concentrations due to it being a P-gp substrate, it might simply be discarded from further development. However, this study showed that such compounds, even though they are likely to be P-gp substrates in humans as well, could reach relatively high total brain concentrations in humans. In fact, [^{18}F]altanserin and [^{11}C]GR205171 were successfully used in humans before they were found to be P-gp substrates. The findings are important for the interpretation of brain drug delivery when extrapolating preclinical data to humans.

In conclusion, pronounced species differences were found in the brain-to-plasma concentrations of three radiolabelled molecules used in PET. It is suggested that the differences observed is a combination of species related differences in P-gp transport,

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potency of CsA to inhibit P-gp and possibly differences in the intra brain distribution of the molecules. Species differences should be considered when extrapolating data obtained in animals to human; compounds found to be P-gp substrates in rodents are likely to also be substrates in higher species but sufficient blood-brain barrier permeability may be retained in humans to allow the compound to act at intracerebral targets.

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Footnotes

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Figure Legends

Figure 1. Chemical structures of the three studied radiotracers. The asterisk represents the radiolabel position.

Figure 2. (A) Brain concentrations (SUV) and (B) brain-to-plasma concentration ratios (K_p) in monkeys (triangles), rats (circles) and guinea pigs (diamonds) after administration of [^{11}C]verapamil. Solid and broken lines represent average values in animals without and with additional CsA administration. Standard errors are shown as y-error bars if $n \geq 3$. SUV and K_p were highest in monkeys, but the increase in brain concentrations after CsA treatment was greatest in rats.

Figure 3. (A) Brain concentrations (SUV) and (B) brain-to-plasma concentrations ratio (K_p) in humans (squares) (Syvänen, 2003), monkeys (triangles), rats (circles) and guinea pigs (diamonds) after administration of [^{11}C]GR205171. Solid and broken lines represent average values in animals without and with additional CsA administration. Standard errors are shown as y-error bars if $n \geq 3$. SUV and K_p were highest in monkeys and humans, but the increase in brain concentrations after CsA treatment was greatest in rats.

Figure 4. (A) Brain concentrations (SUV) and (B) brain-to-plasma concentration ratios (K_p) in humans (squares) (Liptrot et al., 2004), minipigs (triangles) and rats (circles) after administration of [^{18}F]altanserin. Solid and broken lines represent average values in animals without and with additional CsA administration. Standard errors are shown as y-

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error bars if $n \geq 3$. The brain concentrations were highest in minipigs, in which SUV, but not K_p , increased after CsA administration. Both SUV and K_p were increased in rats after CsA.

Figure 5. Sagittal PET images (summation 0-60 min) of [^{11}C]GR205171 in monkey, guinea pig and rat brain without (upper panel) and with (lower panel) CsA administration of CsA 15 mg/kg + 5 mg/kg/h (monkey) or 22.5 mg/kg + 7.5 mg/kg/h (guinea pig and rat) starting 20 min prior to a bolus injection of [^{11}C]verapamil.

Figure 6. Sagittal PET images (summation 0-60 min) of [^{11}C]verapamil in cynomolgus monkey brain without (upper panel) and with (lower panel) administration of CsA 15 mg/kg + 5 mg/kg/h starting 20 min prior to a bolus injection of [^{11}C]verapamil.

Figure 7. Sagittal PET images (summation 0-60 min) of [^{18}F]altanserin in pig brain without (upper panel) and with (lower panel) CsA administration of CsA 22.5 mg/kg + 7.5 mg/kg/h starting 20 min prior to a bolus injection of [^{18}F]altanserin.

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Table 1. Comparison of amino acid content in P-glycoprotein in human, chimpanzee, rhesus monkey, dog, rat, guinea pig and mouse.

Percent of amino acid homology ^a							
P-gp protein ^b	Human	Chimp- anzee	Rhesus monkey	Dog	Guinea pig ^c	Rat	Mouse
Mouse	87	80	85	83	82	93	100
Rat	85	83	88	83	80	100	
Guinea Pig ^c	82	82	82	82	100		
Dog	87	89	87	100			
Rhesus monkey	93	94	100				
Chimpanzee	97	100					
Human	100						

^aThe percentage identity calculated using Ensembl 45 protein sequences (Hubbard et al., 2007) and Blast 2 sequence comparison (Tatusova and Madden, 1999).

^bP-glycoprotein, transcribed from MDR1 (human, chimpanzee, rhesus monkey, dog, guinea pig) and *mdr1a* (rat and mouse) genes.

^cThe whole gene sequence is not yet available. The gene was predicted by projecting human Ensembl transcripts through a BLASTZ DNA alignment to the human genome.

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Table 2. Numbers of subjects for each study and species involved. Numbers in parentheses denote the number of subjects that received cyclosporin A.

	Rat	Guinea pig	Minipig	Monkey	Human
[¹¹ C]verapamil	6 (3)	6 (3)	-	2 (1)	-
[¹¹ C]GR205171	9 (6) ^a	6 (3)	-	5 (1)	5 ^c
[¹⁸ F]altanserine	9 (4) ^b	-	6 (1)	-	5 ^d

^aTwo dose groups of CsA, ^bEx-vivo studies, ^c(Syvänen, 2003), ^d(Liptrot et al., 2004)

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Table 3. Average (standard deviation, $n \geq 3$) baseline SUV and K_p values over 60 min in cerebellum for the studied radiotracers.

	$[^{11}\text{C}]\text{verapamil}$		$[^{11}\text{C}]\text{GR205171}$		$[^{18}\text{F}]\text{altanserin}$	
	<i>SUV</i>	K_p	<i>SUV</i>	K_p	<i>SUV</i>	K_p
Rat	0.21 (0.05)	1.13 (0.36)	0.32 (0.05)	3.45 (0.52)	0.32 (0.11) ^c	0.23 (0.10) ^c
Guinea pig	0.14 (0.02)	0.65 (0.02)	0.20 (0.05)	2.13 (0.83)	-	-
Minipig	-	-	-	-	1.20 (0.14)	2.08 (0.28)
Monkey	0.40 ^a	4.61 ^a	1.57 (0.36)	9.62 (3.93)	-	-
Human ^b	-	-	2.11 (0.84) ^b	29.8 (7.0) ^b	0.90 (0.22) ^d	1.04 (0.49) ^d

^aOne animal, ^b(Syvänen, 2003), ^cEx-vivo studies; values at 60 min after radiotracer administration, ^d(Liptrot et al., 2004)

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Table 4. Average $SUV_{increase}$ and $K_{p,increase}$ over 60 min in cerebellum, striatum and frontal cortex after CsA administration (ns=not significant, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, compared to baseline values)

	$[^{11}C]verapamil$		$[^{11}C]GR205171$		$[^{18}F]altanserin$	
	$SUV_{increase}$	$K_{p,increase}$	$SUV_{increase}$	$K_{p,increase}$	$SUV_{increase}$	$K_{p,increase}$
<i>Cerebellum</i>						
Rat	4.1*	5.8**	3.6**	3.5**	2.6* ^c	2.3* ^c
Rat low dose CsA	-	-	1.4 ^{ns}	1.5**	-	-
Guinea pig	3.9***	2.9**	2.3**	2.0 ^{ns}	-	-
Minipig ^a	-	-	-	-	1.8	0.8
Monkey ^a	4.3	3.6	2.1	2.5	-	-
<i>High binding region^b</i>						
Rat	-	-	-	-	2.6* ^c	2.9* ^c
Minipig ^a	-	-	-	-	2.0	0.9
Monkey ^a	-	-	2.6	2.9	-	-

^aA single animal received CsA

^bHigh binding region: striatum for $[^{11}C]GR205171$ and frontal cortex for $[^{18}F]altanserin$.

^cEx-vivo studies; values at 60 min after radiotracer administration

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Table 5. Average percentage of the tracer not bound to plasma proteins without (w/o) and with (w) cyclosporin A (CsA) administration (standard deviation if n≥3).

	¹¹ C]verapamil		¹¹ C]GR205171		¹⁸ F]altanserin	
	w/o CsA	w CsA	w/o CsA	w CsA	w/o CsA	w CsA
Rat	5.5 (1.9)	6.2 (2.2)	10.5 (0.5)	13.0 (0.6) ^b	<1	<1
Guinea pig	3.5 (1.8)	3.6 (1.4)	9.7 (3.0)	13.9	-	-
Minipig	-	-	-	-	<1	<1
Monkey 1	-	-	11.3, 9.4 ^a	9.6	-	-
Monkey 2	8.4, 8.5 ^a	7.9	11.0	-	-	-
Human	-	-	14.9 (1.6)	-	<1	-

^atwo scans were performed in the same animal. The second value was determined in plasma obtain 1-2 min before CsA administration.

^bsignificantly (p<0.001) higher with CsA than without according to a one-tailed t-test.

Figure 1

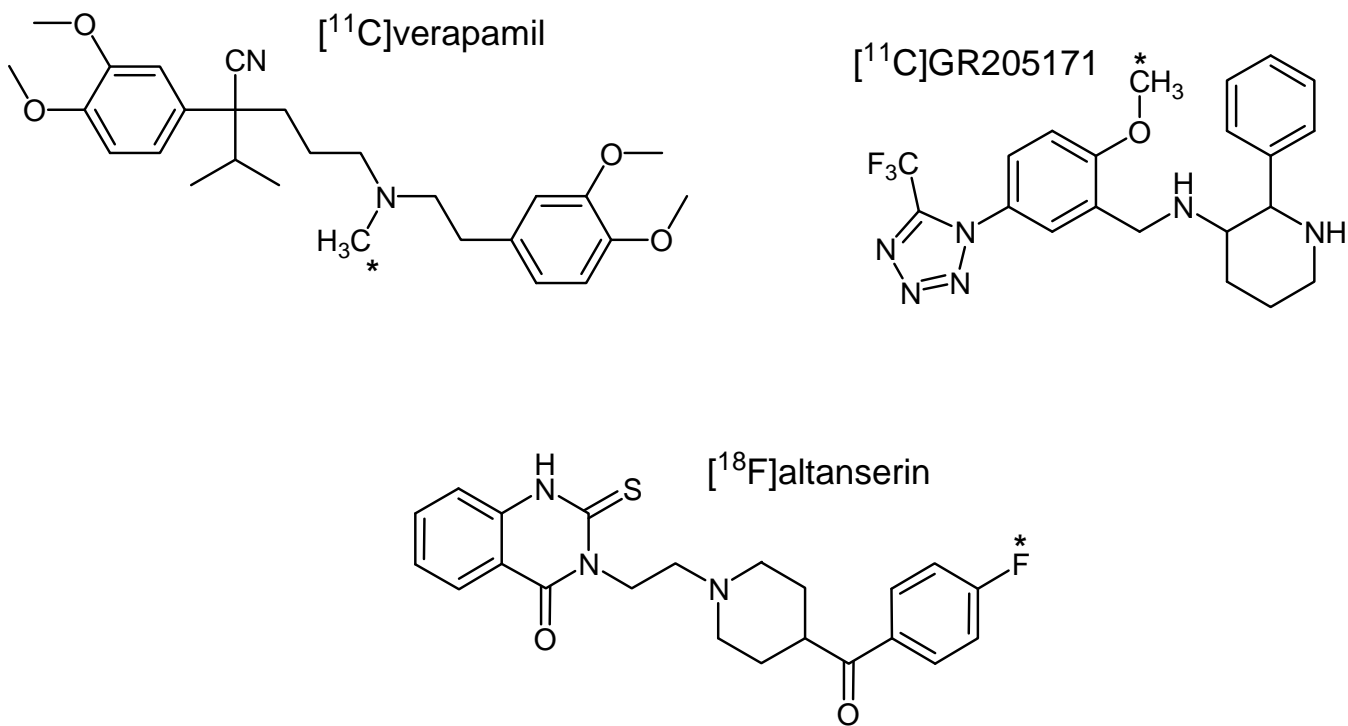


Figure 2

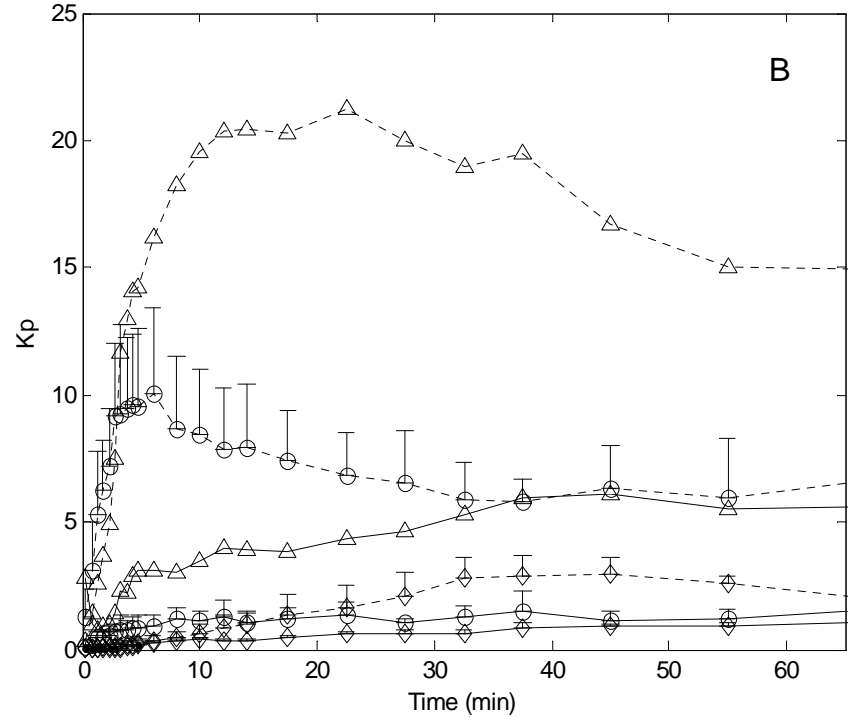
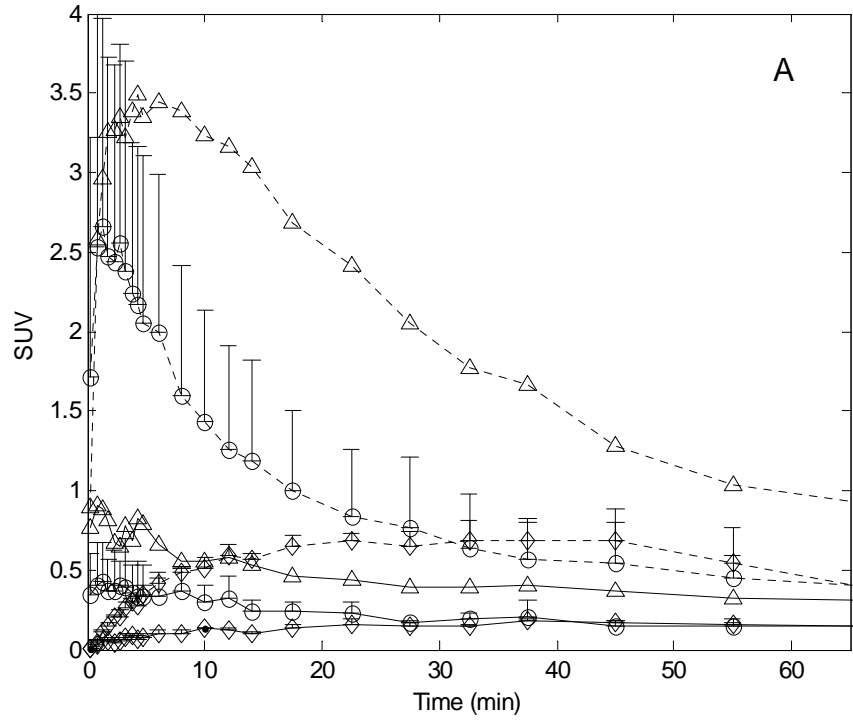


Figure 3

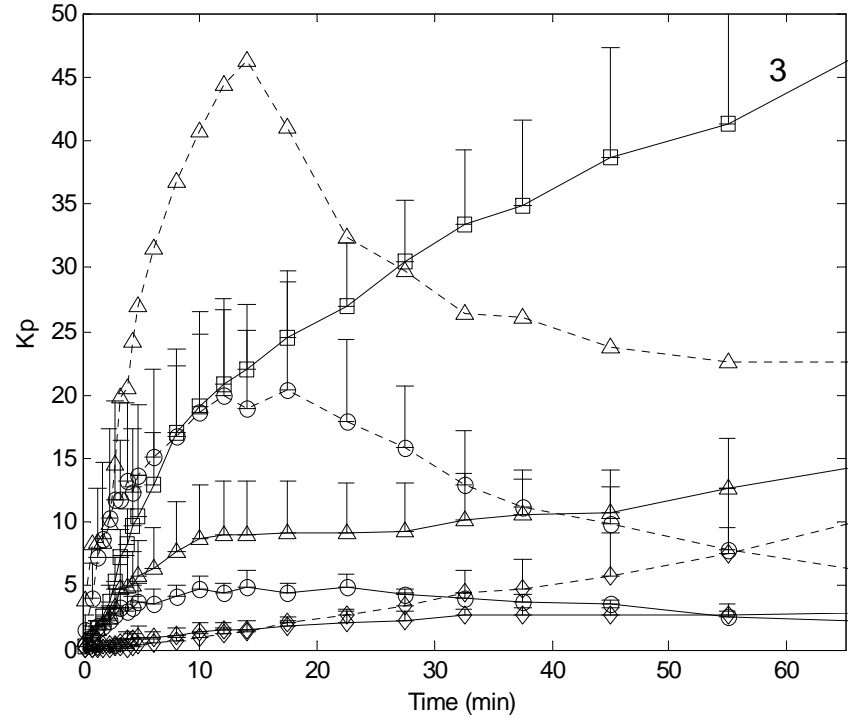
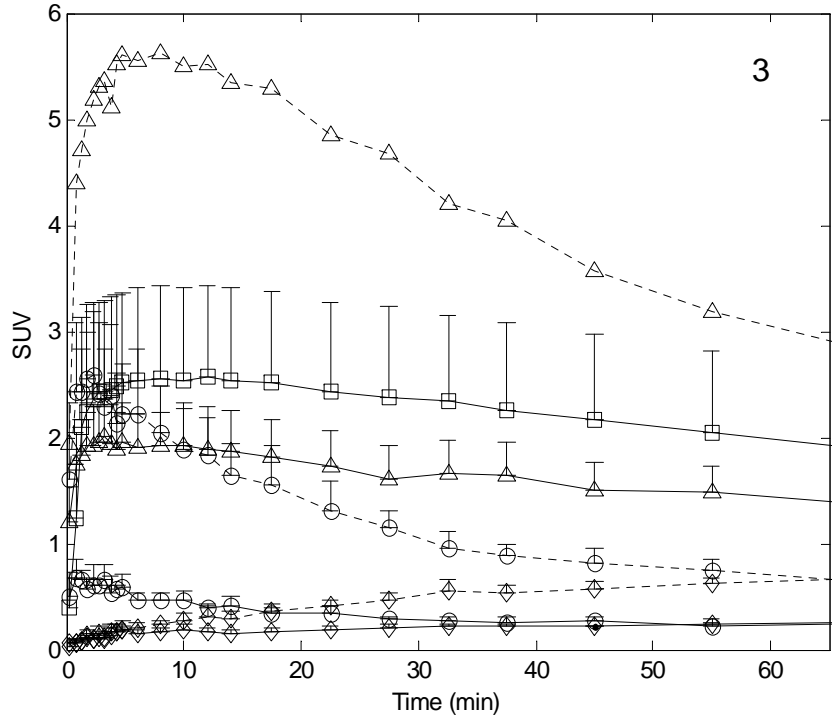


Figure 4

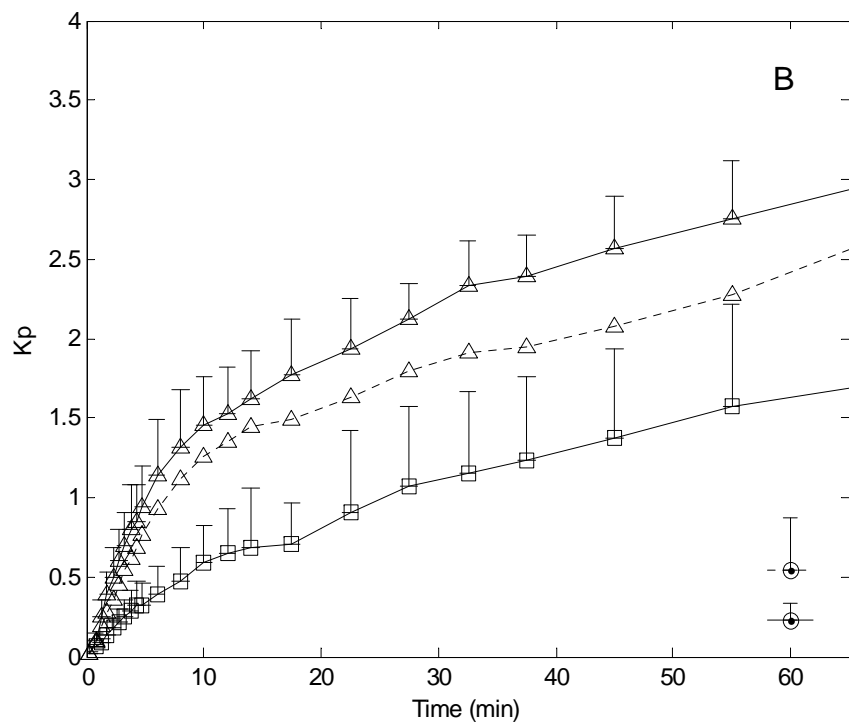
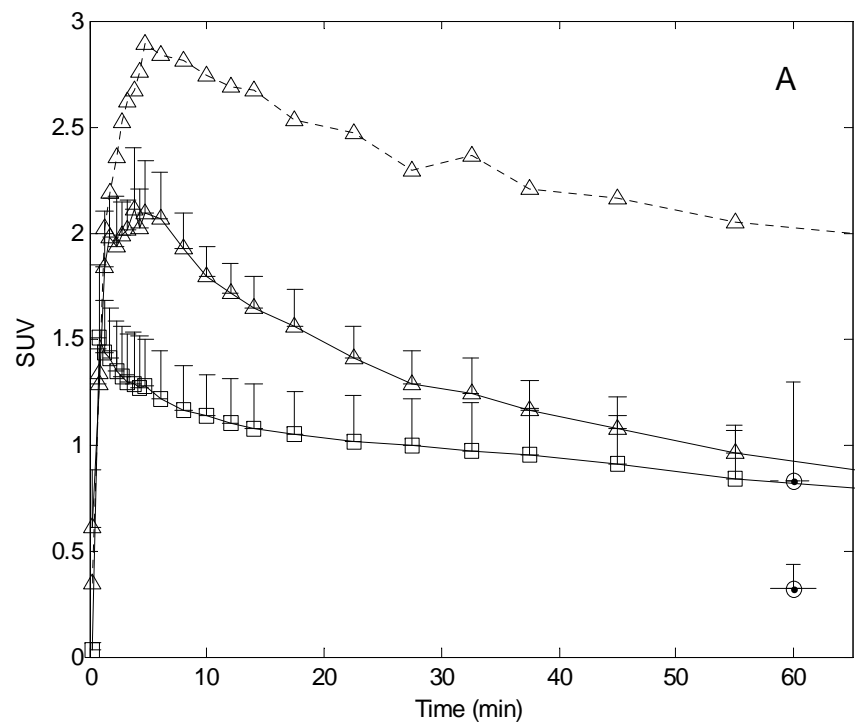


Figure 5

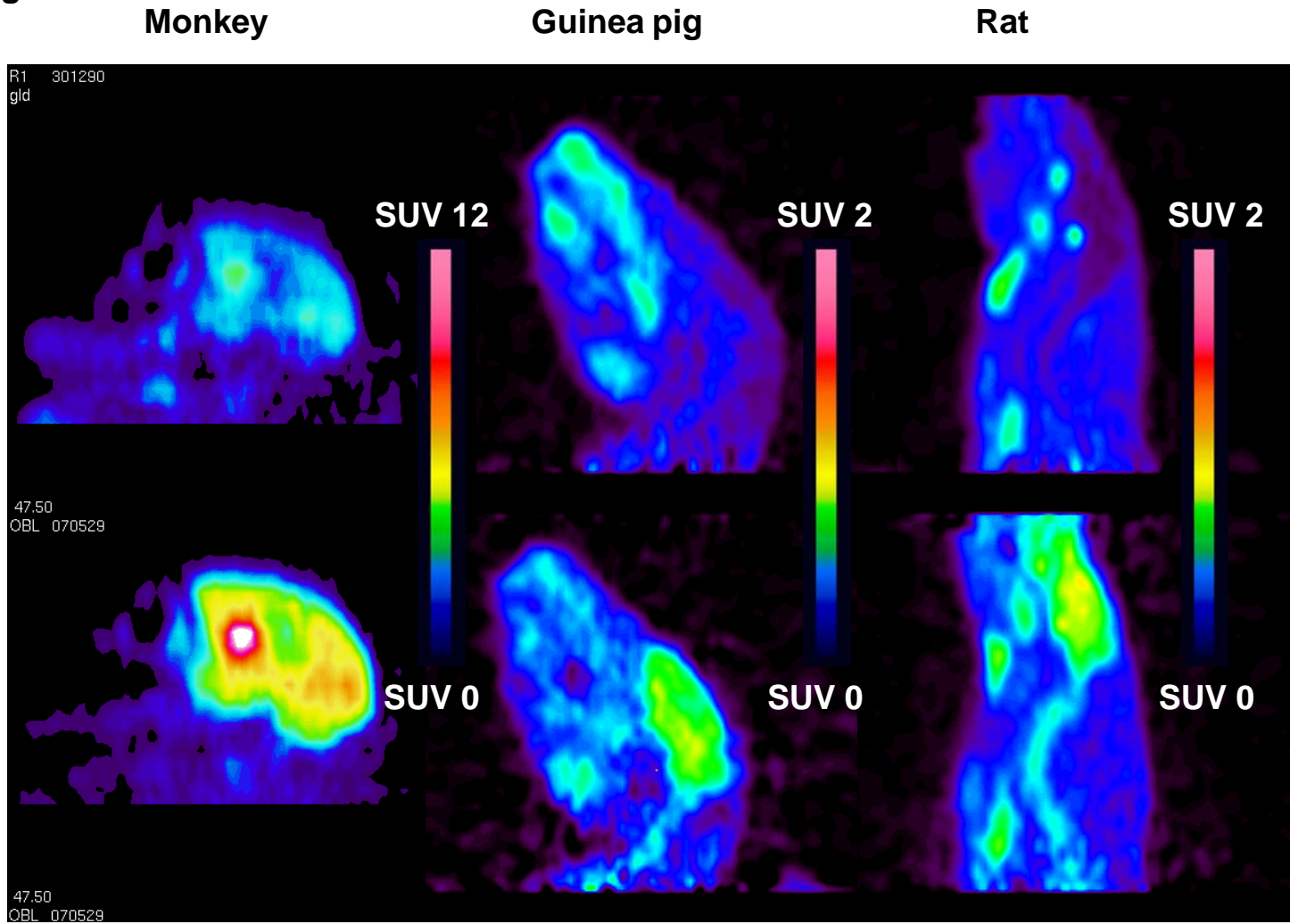


Figure 6

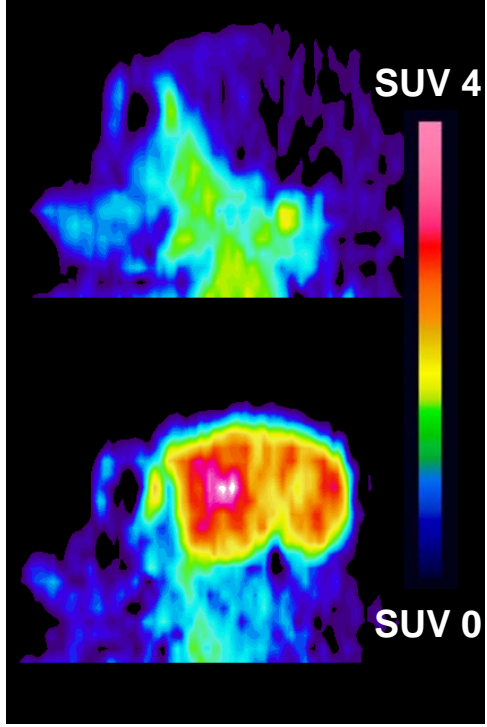


Figure 7

