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**P-GLYCOPROTEIN AND MUTLIDRUG RESISTANCE ASSOCIATED
PROTEINS LIMIT THE BRAIN UPTAKE OF SAQUINAVIR IN MICE.**

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Running Title: P-gp and Mrps Limit the Brain Uptake of Saquinavir in Mice

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Abbreviations:

SQV, saquinavir; P-gp, P-glycoprotein; Mrps, multidrug resistance associated proteins;

HIV, human immunodeficiency virus; PI, protease inhibitor; AIDS, Acquired Immune

Deficiency Syndrome; V_{vasc}, brain vascular volume; V_{brain}, apparent brain distributional

volumes; K_{in}, the unidirectional transfer coefficient.

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Abstract

Efflux transporters such as P-glycoprotein (P-gp) and multidrug resistance associated proteins (Mrps) and their contributions to saquinavir (SQV) brain uptake were characterized. Cerebral flow rate was estimated from diazepam uptake and brain vascular volume was assessed using inulin. Mice brains were perfused with buffer containing SQV alone or co-perfused with different concentrations of GF120918, a P-gp inhibitor or MK571, a specific Mrp family inhibitor. Inulin, a nonabsorbable marker, was also co-perfused in all studies to assess if the inhibitors altered the physical integrity of the BBB. The estimated cerebral flow rate using diazepam was 250 ml·100g^{-1}·min^{-1}. The brain vascular volume, estimated using inulin, was almost constant (0.94 ± 0.03 ml·100g^{-1}, n=12) during the perfusion study. SQV uptake kinetics were linear during the sampling period. Inclusion of 10 µM GF-120918 in the perfusate resulted in a more than 7-fold increase in the brain distributional volume (i.e., uptake) of SQV. Inclusion of 100µM MK571 in the perfusate increased SQV apparent brain uptake by more than 4.4-fold suggesting, for the first time, that Mrp transporters may play an important role in the brain uptake and retention of SQV. Neither GF-120918 nor MK571 altered the integrity of the BBB during the time course of the study. While the current results reaffirm that SQV is a P-gp substrate, this is the first report implicating the Mrp transporter family in the limited brain uptake and retention of SQV *in vivo* in mice.

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Introduction

The HIV-1 protease inhibitor (PI), saquinavir (SQV), was the first agent among human immunodeficiency virus (HIV) protease inhibitors (PI) approved and used for the treatment of Acquired Immune Deficiency Syndrome (AIDS) and has very high selectivity and binding affinity to HIV-1 Protease (Martin, 1992). Combination therapies using protease- and reverse transcriptase- inhibitors have proven successful in reducing the plasma viral load of HIV-infected patients (Collier et al., 1996). However, SQV has a number of biopharmaceutical limitations, related in many instances to poor and/or variable transport across important biological membranes. For example, oral absorption is often low and variable and penetration into the brain is very poor (Aungst, 1999). Several factors including poor solubility, high protein binding, and extensive first pass metabolism contribute to poor transport across biological membranes (Barry et al., 1997). In addition, secretory membrane transporters including P-gp and multidrug resistance-associated proteins (Mrps) have been implicated from *in vitro* studies (Kim et al., 1998;Williams et al., 2002). The expression of P-gp in capillary endothelial cells of the brain has been shown to be a critical factor in preventing the entry of SQV into the central nervous system (CNS) (Kim et al., 1998). CNS drug therapy is very challenging since few CNS-active drugs have appreciable permeability across the BBB (Bickel et al., 2001). Since the CNS represents a sanctuary site for HIV-1 infection, the incidence of CNS complications from secondary pathologies such as AIDS dementia is high (Gendelman et al., 1997). Hence, the poor permeation of SQV into the brain is likely to limit the effective treatment of AIDS.

The blood-brain barrier (BBB), composed of a single layer of endothelial cells connected by tight junctions, restricts the transport of compounds from the circulating blood into the brain (Pardridge, 1995). The poor distribution of drugs into the CNS is also limited by specific membrane efflux transporters such as P-gp, the product of the multidrug resistance (MDR) gene (Kakee et al., 1996). Besides P-gp, which is expressed on the luminal side of endothelial cells, other ATP-binding cassette (ABC) transporters such as the family of Mrps (Borst et al., 2000) and breast cancer resistance protein (BCRP) (Eisenblatter and Galla, 2002) have been found in human and rodent brains. At this time, the functions and locations of the Mrps and BCRP in the brain have not been fully clarified.

Using an immunostaining method Miller et al. showed that P-gp and Mrp2 were expressed in the luminal side of fish, rat, and pig brain capillaries (Miller et al., 2002;Miller et al., 2000) whereas mRNA encoding Mrp2 in bovine brain capillary endothelial cells and rat brain capillaries has not been detected by other investigators (Zhang et al., 2000;Sugiyama et al., 2003). Mrp1 has been detected on bovine and rat brain capillary endothelial cells (Huai-Yun et al., 1998;Regina et al., 1998) and it has been also found on the basolateral side of epithelial cells in the choroid plexus (Rao et al., 1999). The mRNA encoding Mrp1, Mrp4, Mrp5, and Mrp6 in both bovine brain capillary endothelial cells and capillary homogenates were detected (Zhang et al., 2000). Nevertheless, the function of Mrp homologues in brain capillary endothelial cells in controlling the transport of drugs across the BBB remains to be investigated.

Transport studies using an *in vitro* model of the BBB suggested that the expression of P-gp (Glynn and Yazdanian, 1998) and Mrps was functional. However,

since the expression of these efflux transporters may be up- or down- regulated during cell culture (Gutmann et al., 1999b), *in vivo* investigations into the role of the efflux transporters in facilitating SQV brain uptake and retention are required to fully understand the possible role of these proteins in the BBB.

The aim of this study was to elucidate the mechanism responsible for the low brain uptake of SQV using an *in situ* mice brain perfusion technique. The contribution of efflux transporters such as P-gp and Mrps to brain uptake of SQV was characterized by directly perfusing mice brains with a physiological buffer including SQV in the absence and presence of selective inhibitors of P-gp and Mrps.

Materials and Methods

Materials

[^{3}H] inulin (0.54 Ci/mmol) and [^{14}C] diazepam (56 mCi/mmol) were purchased from Amersham Biosciences (UK). [^{14}C] saquinavir (26.5 µCi/mg) was provided by Roche Discovery Welwyn (Welwyn Gardon City, UK). GF120198 and MK571 (base form) were provided by GlaxoSmithKlein, Inc. (Research Triangle Park, NC) and Merck Laboratories, Inc. (Whitehouse, NJ), respectively. D-Glucose was purchased from Sigma. Solvable$^®$ was purchased from Packard Instruments (Downers Grove, IL). All other chemicals and reagents were purchased from Fischer Scientific (Houston, TX) and were of analytical grade.

Animals

Male FVB mice (26-30g) were purchased from Taconic (New York, USA) and maintained under standard conditions of temperature and lighting with *ad libitum* access

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to food and water. All experimental procedures complied with the protocol (02-029)

approved by the Institutional Review Board Use and Care of Animal Committee, and

housed in AAALAC accredited facilities at Rutgers University.

In situ brain perfusion

The *in situ brain* perfusion technique used in the experiments was similar to that

described elsewhere (Takasato et al., 1984;Dagenais et al., 2000) with modifications.

Briefly, for surgical preparation, mice were anesthetized with ketamine/xylazine (140/8

mg/kg, i.p.). After exposure of the left common carotid artery, the left external carotid

artery was ligated at the bifurcation of the common carotid artery with the internal carotid

artery. The left common carotid artery was ligated caudally. A polyethylene tube (0.30

mm i.d. $\times$ 0.70 mm o.d.) filled with heparin (20 units/ml) was catheterized into the left

common carotid artery under the microscope. For perfusion experiments, the left

hemisphere of the mouse brain was perfused with perfusion buffer containing test

compounds at a selected flow rate through the catheter that was connected to the

perfusion pump. The perfusion buffer consisted of bicarbonate buffered physiological

saline (128 mM NaCl, 4.2 mM KCl, 24 mM $NaHCO_3$, 2.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$,

and 0.9 mM $MgSO_4$). D-glucose (9 mM) was added prior to an experiment. The

perfusate was bubbled with a mixture of 95% O_2 and 5% CO_2 for pH control (7.4) and

maintained at 37 °C. Immediately prior to initiation of the perfusion, the cardiac

ventricles were severed to eliminate the contribution of contralateral blood flow.

Multiple time point experiments (15, 30, 60, and 90 s for diazepam and 30, 60, 90, and

120 s for SQV) were performed at a calibrated flow rate (Harvard pump PHD2000;

Harvard apparatus, Holliston, MA, U.S.A.). At least 0.8 µCi/ml of [^{3}H] inulin was added

into the perfusates as a vascular space marker to obtain about 1000 dpm/tissue sample.

The perfusates contained radiolabeled [^{14}C] diazepam or [^{14}C] SQV with or without

GF120918 or MK571 to produce an appropriate drug concentration. The stock solutions

of GF120918 (4mM) and MK571 (13mM) were prepared in DMSO. The stock solutions

were diluted with the bicarbonate-buffered saline used for perfusion.

The perfusion was terminated by decapitation. The brain was removed from the skull,

dissected and each hemisphere was weighed, and placed in a pre-weighed scintillation

vial. Brain and perfusion fluid samples were digested in 0.7 ml of Solvable (Packard

BioScience) at 37°C for 24 hours. Scintillation cocktail (5ml) was added to each vial and

radioactivities of [^{3}H] and [^{14}C] were determined simultaneously by dual liquid

scintillation counting. All data are reported for the left hemisphere.

Calculations and Data analysis.

Data from the brain perfusion method was analyzed as described previously (Dagenais et

al., 2000;Chen et al., 2002). Briefly, brain vascular volume (V_{vasc}, ml·100g^{-1}), defined as

the ratio of the vascular marker concentration in brain to that in the perfusate, was

determined using the following equation:

$$V_{vasc} = X^*/C^* \qquad (1)$$

where X^* is the amount of radiolabeled inulin in the brain (dpm · 100 g^{-1}) and C^* is the

perfusate concentration (dpm· ml^{-1}). The unidirectional transfer coefficient K_{in} (ml · min^{-1}·100 g^{-1}) was calculated using the following relationship:

$$K_{in} = dX_{brain}/dt/C_{pf} \qquad (2)$$

where X_{brain} is the amount of radiotracer in the brain (dpm $\cdot$ 100 g^{-1}) corrected for

vascular contamination ($X_{total} - V_{vasc} \cdot C_{pf}$), X_{total} (dpm$\cdot$ 100 g^{-1}) is the total quantity of

tracer measured in the tissue sample (vascular + extravascular), and C_{pf} is the tracer

concentration in the perfusate (dpm $\cdot$ ml^{-1}). In a single time point experiment, X_{brain}/T

replaced dX_{brain}/dt, where T was the perfusion time (minutes). Apparent brain

distributional volumes (V_{brain}, ml$\cdot$ 100 g^{-1}) were calculated from

$$V_{brain} = X_{brain}/C_{pf} \tag{3}$$

Brain flux J (nmol/g brain/min) was calculated according to:

$$J = K_{in} \cdot C_{pf} \tag{4}$$

where C_{pf} is the SQV concentration in the perfusate. Kinetic relationships between the

concentration dependency of SQV uptake were described by a nonlinear least-squares

method. SQV brain flux J was divided into two components:

$$J = J_d - J_{efflux} \tag{5}$$

where J_d is the maximal brain flux of SQV when no efflux transporters are involved and

it is transported by passive diffusion depending on the physicochemical properties of the

drug and J_{efflux} is the transporter-mediated component (i.e., efflux transporters at the

luminal membrane of the BBB endothelial cells). The best-fit line was obtained using a

modified Michaelis-Menten equation as follows (GraphPad Prism4, San Diego, CA,

U.S.A.):

$$(J = K_d \, C_{pf} - [J_{max} \, C_{pf} /(K_m + C_{pf})] \tag{6}$$

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where J_{max} is the maximum uptake rate for a saturable component, K_m is the Michaelis

constant, K_d is the first-order constant for the non-saturable component and C_{pf} is the

concentration of SQV in the perfusates. SQV uptake data at 60 seconds in the inhibition

studies were described using the following relationship:

$$K_{in} = (K_{in, max} \cdot C) / (IC_{50} + C) \tag{7}$$

where $K_{in, max}$ is the maximal brain K_{in} (ml $\cdot$ min$^{-1} \cdot$100 g^{-1}), C is the inhibitor

concentration in the perfusate (μmol/L), and IC_{50} is the inhibitory concentration at half-

maximal brain K_{in} (μmol/L). Estimates of SQV inhibition study parameter (IC_{50}) were

obtained by fitting a Michaelis-Menten equation to the unidirectional transfer coefficient

K_{in} versus concentrations of inhibitors data by nonlinear least-squares regression using

the same software. All the graphs were made using Excel2000 and GraphPad Prism4.

Data are presented as mean $\pm$ SEM for three to five animals. When appropriate, ANOVA

or two sided Student t tests were used to determine the statistical significance of

differences between experimental groups. Statistical significance was determined at the

level of $p = 0.05$.

Results

Determination of the perfusion conditions.

In order to study mechanisms restricting brain uptake of SQV, the perfusion conditions

were first validated and optimized by investigating brain uptake of radiolabeled markers

such as [^{14}C] diazepam and [^{3}H] inulin. [^{14}C] diazepam (0.3 μCi/ml) was used for

selecting an appropriate perfusion flow rate and estimating regional cerebral perfusion

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rate since diazepam is known to be completely extracted during a single pass through the

brain and has no significant back flow (Takasato et al., 1984). All perfusates contained

[^{3}H] inulin (1 μCi/ml) as a vascular marker because it does not significantly penetrate the

BBB during short periods of perfusion. A linear relationship between initial brain transfer

coefficient (K_{in}) of diazepam at 20 seconds and perfusion flow rate was observed (Fig.

1A). A perfusion flow rate of 2.1 ml · min^{-1}, which results in K_{in} of diazepam similar to

that in a previous report in mice (Dagenais et al., 2000) was chosen and used for the rest

of experiments. The integrity of the BBB was checked by monitoring the distribution

volume of [^{3}H] inulin (V_{vasc}) for each mouse perfused. BBB integrity was well

maintained at the higher flow rates. The regional cerebral fluid flow (F_{pf}; ml · g^{-1} · s^{-1})

was estimated in a separate perfusion experiment at 2.1 ml · min^{-1} (15-. 30-, 60, and 90-

second time points). As shown in Fig. 1B, [^{14}C] diazepam showed linear uptake kinetics

through 90 seconds with a K_{in} of 250 ml ·100g^{-1} · min^{-1} (Fig. 1B). This observation is

also consistent with previously reported results (Dagenais et al., 2000). The brain

vascular volume was estimated using [^{3}H] inulin and it was constant (0.94 ± 0.03 ml· 100

g^{-1}; means $\pm$ S.E.M., n = 12) during perfusion for 15-90 sec (Fig. 1B). A control

experiment was performed in order to evaluate the effect of DMSO that was used for the

preparation of stock solutions of inhibitors. The brain uptake of SQV was not affected by

up to 4% of DMSO (data not shown).

Time course of SQV uptake

The brain uptake of [^{14}C] SQV in mice was studied using the *in situ* mouse brain

perfusion technique in order to choose a reliable perfusion time that allows for sufficient

drug accumulation in the brain tissue (Fig. 2). Based on the results of the validation

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studies, a perfusion flow rate of 2.1 ml/min was selected for these experiments. The

relationship between SQV brain uptake and perfusion time for up to 120 seconds was

linear while no BBB disruption was observed (●, V_{vasc} of [^{3}H] inulin). The radioactivity

was about 4000 dpm/tissue sample at 60s of perfusion time when the perfusate contained

0.3 μCi/ml of [^{14}C] SQV. Since sufficient accumulation of [^{14}C] SQV was obtained after

60s of perfusion, this perfusion time was chosen for all subsequent studies.

Concentration dependency of SQV uptake

The mice brains were perfused with various concentrations of SQV (1-36 μM) in order to

determine whether the brain uptake of SQV was concentration dependent. Fig. 3. shows

the rate of SQV uptake in the left hemisphere as a function of SQV concentration in the

perfusate. As the concentration of SQV increased, the brain uptake rate of SQV increased

steeply. The unidirectional transport coefficient (K_{in}) was significantly changed from

4.3 ± 0.2 ml · min^{-1}·100 g^{-1} at 13 μM to 9.7 ± 0.8 ml · min^{-1}·100 g^{-1} at 36 μM of SQV (K_{in}

value; means ± S.E.M., n = 4). The best-fit line ($R^2 = 0.97$) was obtained using a

modified Michaelis-Menten equation as described under *Calculations and Data analysis.*

Effect of GF120918 on the brain uptake of SQV

HIV protease inhibitors such as SQV have shown very limited oral absorption and brain

entry and the major efflux transporter present in the BBB has been reported to be P-gp

(Regina et al., 1998;Zhang et al., 2000). Therefore, this experiment was performed to

affirm the role of P-gp on the brain permeation of SQV using the perfused brain model.

To determine the effect of GF120918 on the brain uptake of SQV, mice brain perfusion

studies of SQV were performed in the absence and presence of various concentrations of

GF120918. The V_{brain} value for SQV increased significantly as the concentration of

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GF120918 in the perfusate increased from 0 to 20 μM. The V_{brain} values eventually

plateaued at GF120918 concentrations greater than 10 μM ($\Diamond$, Fig. 4). Inclusion of GF-

120918 (10 μM) in the perfusates inhibited P-gp activity in the BBB and significantly

increased the V_{brain} values of SQV by more than 7-fold. When the V_{brain} values of SQV in

various concentrations of GF120919 were fitted to a modified Michaelis-Menten

equation, an IC_{50} value of 2.69 $\pm$ 0.82 μM was obtained. The brain vascular volume ($\bullet$,

V_{vasc} of [^{3}H] inulin, Fig. 4) during perfusion remained constant (1.24 $\pm$ 0.02 ml$\cdot$ 100 g^{-1};

means $\pm$ S.E.M., n = 25). This result demonstrated that the presence of GF120918 did not

alter the integrity of the tight junctions.

Effect of MK571 on the brain uptake of SQV.

Mrps and P-gp have many substrates in common including the HIV protease inhibitors.

Previous reports from our lab and others suggest that Mrp2 efficiently transported SQV

in overexpressed Mrp2 *in vitro* cell culture models (Huisman et al., 2002;Williams et al.,

2002). We evaluated the brain uptake characteristics of SQV in the presence of various

concentrations of MK571, a specific Mrp transporter family inhibitor using the brain

perfusion technique in order to investigate the *in vivo* contribution of Mrp on brain uptake

of SQV. Inclusion of MK571 (100 μM) in the perfusates increased the V_{brain} values of

SQV by more than 4.4- fold compared to SQV alone ($\Diamond$ in Fig. 5). When the V_{brain} values

of SQV in the absence of presence of MK571 were fitted to a modified Michaelis-Menten

equation, an IC_{50} value of 7.23$\pm$4.58 μM was obtained. Data are mean $\pm$ S.E.M. (n=3~4).

As shown in the graph ($\bullet$, V_{vasc} of [^{3}H] inulin=1.25 $\pm$ 0.03 ml$\cdot$ 100 g^{-1}; means $\pm$ S.E.M., n

= 20), addition of MK571 in the perfusates did not alter the integrity of the tight junctions.

Discussion

The mouse or rat brain perfusion technique is a very useful tool for studying the brain distribution of xenobiotics (e.g., drugs) since, unlike *in vitro* models, drug transport across the BBB is measured in the intact brain with its full complement of enzymes, proteins and transport systems. Along with the "leakiness" of cultured brain endothelial cells, any difference or change in expression of xenobiotic transporters compared to *in vivo* conditions (e.g., down-regulation of some nutrient transporters (Pardridge, 1995) or up-regulation of Mrp (Gutmann et al., 1999b)) make *in vitro* to *in vivo* extrapolation difficult. Compared to typical *in vivo* biodistribution studies that assess the amounts of brain "associated" drug (i.e., adsorbed to tissues, in the brain vasculature, etc.), *in situ* perfusion methods measure actual drug uptake. The *in situ* brain perfusion technique has been used to investigate the mechanisms of transport of various drugs across the BBB (Smith, 1996). We validated the *in situ* perfused mice brain model before performing the studies to elucidate the mechanisms of brain uptake of SQV. A perfusion rate of 2.1 ml/min, at which a similar K_{in} value for diazepam was obtained in a previous report (Dagenais et al., 2000), was chosen since the physical integrity of the BBB was also maintained. [^{14}C] Diazepam permeates the BBB by passive diffusion via the transcelluar route (Takasato et al., 1984) and the results of kinetic studies (Fig. 1B) confirmed linear diazepam brain uptake. Monitoring the brain vascular volume using vascular markers such as inulin assesses the integrity of the BBB during brain perfusions. The brain vascular volume of [^{3}H] inulin did not change significantly during perfusion for 20-90 sec and was comparable to the values (1.1 ml $\cdot$ 100 g^{-1} in the mouse (Murakami et al., 2000) and 6-9 ml $\cdot$ 100 g^{-1} in the rat (Takasato et al., 1984)) that were previously reported.

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Furthermore, in all experiments, the brain distribution volume of [^{3}H] inulin remained low (1.3 ± 2 ml $\cdot$ 100 g^{-1}) in the perfusates containing SQV in the absence and presence of GF120918 or MK571, indicating that the integrity of the BBB was maintained during the perfusion.

The brain uptake of SQV was linear and unidirectional without any measurable disruption of the BBB for at least 120s during perfusion. Since linearity was maintained the possibility of underestimating brain SQV uptake was minimized. Potentially confounding systemic disposition effects (e.g., metabolism and protein binding) are avoided by directly perfusing the drug solution into the brain hemisphere. This was especially important for SQV since it shows high plasma protein binding (> 98% (Kim et al., 1998)) and extensive first pass metabolism. Therefore, the extent of brain uptake can be evaluated by directly assessing apparent brain distribution volume. The apparent brain distribution volume of SQV is low (4.3 ml $\cdot$ 100 g^{-1} at 60s) despite its favorable physicochemical properties (e.g., high lipophilicity with log P=4.4 and molecular weight=670.9). Under the same experimental conditions used in this study, the maximal possible value for K_{in} was estimated to be 250 ml$\cdot$ 100 g$^{-1}\cdot$ min^{-1}.

Most of the published SQV brain uptake studies utilize traditional pharmacokinetic (PK) techniques in mice with a specific knocked out gene (e.g., mdr1). Brain concentrations were typically assessed by examining the total amount of SQV associated with brain tissue and then compared to a wild type mice control. There are many shortcomings to this type of study design. For example, brain uptake can be overestimated since all tissue-associated SQV (i.e., bound to tissue, in the vascular space, etc.) is considered "absorbed". In cases where assay sensitivity is not an issue, traditional

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PK studies can detect substantive differences in brain uptake but only in a semi-quantitative manner. However, the issues of compensatory changes in the cytochromes P450 and the expression of other transporters in knock out animal models can obscure the interpretation of results (Cisternino et al., 2004;Schuetz et al., 2000) even further. In this report, the SQV brain transport mechanisms were elucidated in a quantitative manner allowing for the observation of a novel transport mechanism. As shown in Fig. 3, transport rate of SQV in the left hemisphere did not increase proportionally to the concentration of SQV in the perfusate. The unidirectional transport coefficient increased by more than 2-fold at the highest concentration of SQV (36 μM) as compared to no significant increase in the lower concentration range (1-13 μM). This greater than proportional increase in SQV brain uptake at higher concentrations is consistent with the saturation of a secretory transporter(s) or a metabolic enzyme since saturation leads to a greater rate of uptake. A similar relationship between relative oral absorption and dose was established following the administration of single doses of SQV, ritonavir, indinavir, nelfinavir and amprenavir in humans. The relationship showed greater than dose proportional increases in oral absorption with SQV showing the highest non-linearity among the HIV PIs (Williams and Sinko, 1999). This is in contrast to saturation of an absorptive transporter system where the rate of uptake is lower at higher concentrations (Sinko and Amidon, 1988).

Of the potential mechanisms responsible for this behavior, P-gp has been implicated by our group and others from *in vitro* studies (Polli et al., 1999;Williams et al., 2003) and from semi-quantitative *in vivo* studies (Sparreboom et al., 1997). However, the importance of other efflux transporters on SQV brain uptake has not been conclusively

demonstrated *in vivo*. Recent studies have demonstrated that the brain penetration of the

HIV PIs such as indinavir, nelfinavir and SQV is markedly affected by mdr1a P-gp since

they are excellent substrates for this transporter (Kim et al., 1998;Lee et al., 1998). In

addition, it was observed in rats that the distribution ratio of SQV in plasma: brain:

cerebrospinal fluid was approximately 100:10:0.2 (Washington et al., 2000). Recently,

BCRP (ABCG2), a member of the ABC family of drug transporters, was found to be

highly expressed in the placenta and the luminal surface of the microvessel endothelium

of the BBB where it may play a role in limiting the penetration of drugs. However, little

is known about the function of BCRP in the BBB *in vivo* (Cisternino et al., 2004). For the

purpose of this study, however, the role of BCRP in SQV brain uptake was considered

minimal since Gupta et al. recently reported that HIV PIs including SQV were not

substrates for BCRP (Gupta et al., 2004).

Although the role of P-gp for limiting the brain entry of xenobiotics is well

established (Schinkel, 1999), the role of the Mrps is much less well understood. The

current results show that Mrps represent a significant permeation barrier for SQV across

the BBB *in vivo*. Unlike P-gp, the role of the Mrps in BBB function should be considered

with respect to differences in cellular localization of the various family members. Mrp2 is

located on the apical cell membrane whereas other Mrps including Mrp1, Mrp3, and

Mrp5, are located at the basolateral side (Borst et al., 2000). Although MK571is not

specific for Mrp2, (i.e., it also inhibits Mrp1), it is reasonable to use as an inhibitor of

Mrp2 in drug transport studies, especially in tissues such as brain capillary endothelial

cells since Mrp1 is not predominantly expressed (Regina et al., 1998). In addition, SQV

is known to be a substrate for Mrp2 *in vitro* in cell culture models overexpressing the

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transporter (Huisman et al., 2002;Williams et al., 2002;Gutmann et al., 1999a). Therefore, increased brain uptake of SQV in the presence of MK571 is likely due to Mrp2.

In summary, results obtained in this study suggest that the *in situ* mouse brain perfusion model is a suitable technique in assessing the interaction of SQV with efflux transporters such as P-gp and Mrps *in vivo*. The finding that SQV is subject to transport by P-gp and Mrps in the brain suggests that P-gp and Mrps have an overlapping substrate spectrum, which is known from substrate recognition studies on P-gp and Mrps (Lee et al., 2001). Furthermore, our results suggest that increasing the low brain uptake of SQV may require parallel strategies for overcoming the effect of both P-gp and Mrp2.

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Legends for Figures:

Fig. 1. (A) Unidirectional transfer coefficient K_{in} of diazepam as a function of perfusion rate. A linear relationship between diazepam K_{in} in the left hemisphere at 20 seconds and perfusion flow rate was observed. Data are presented as mean ± S.E.M. (n=3 ~ 4).

(B) Time course of diazepam uptake, expressed as apparent brain distributional volume, V_{brain}, for various perfusion time (15, 30, 60, 90 seconds) at perfusion flow rate 2.1 ml/min. The solid line is the linear regression of the mean V_{brain} values at each time point. ($r^2 = 0.994$). Data are presented as mean ± S.D. (n=3 ~ 4). Estimated cerebral flow rate using [^{14}C]diazepam was 250 ml·100g^{-1}·min^{-1}.
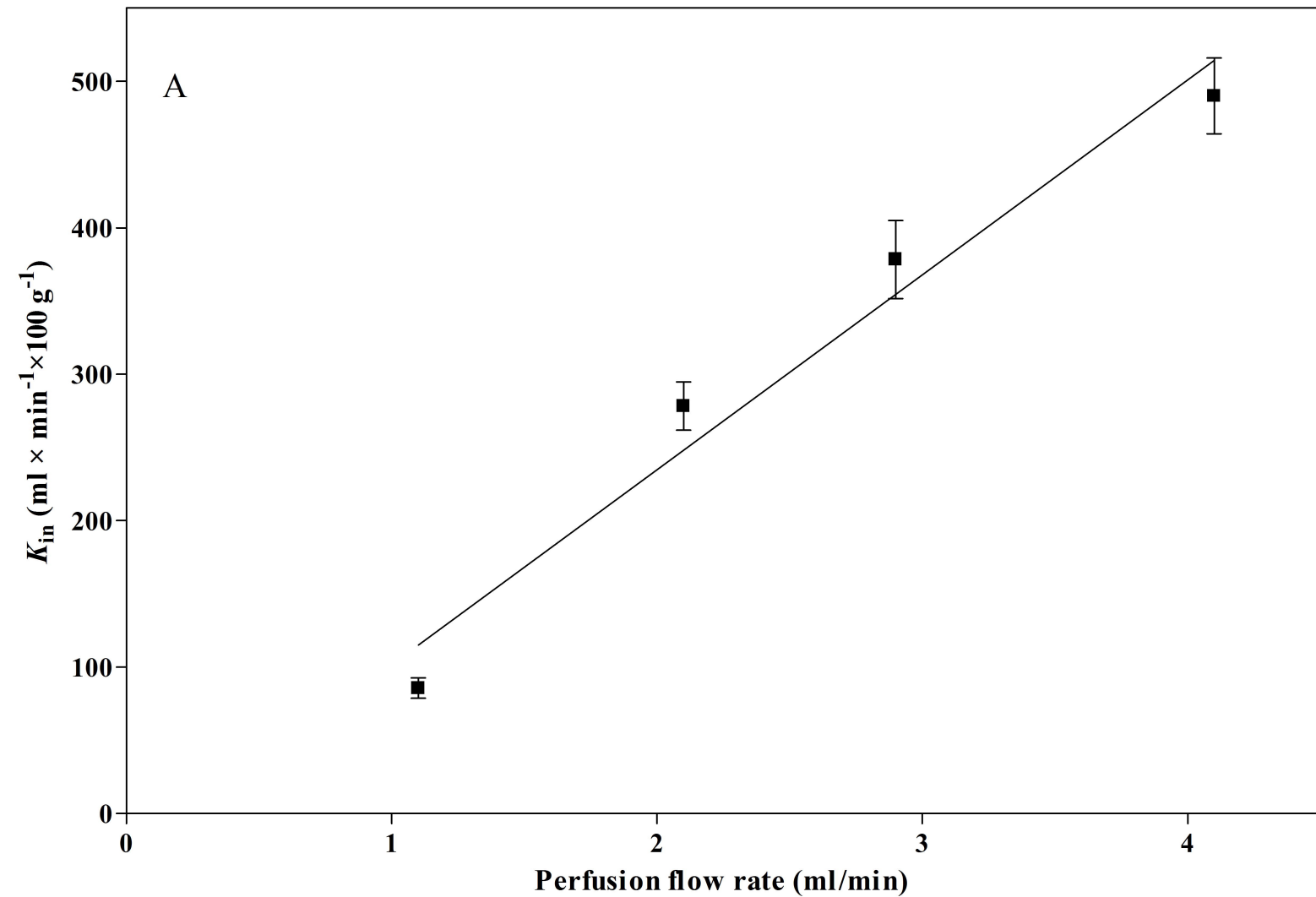
Fig. 2. Time course of brain uptake of [^{14}C] SQV (◊) and [^{3}H] inulin (●) was evaluated by the *in situ* mice brain perfusion for various perfusion time (30, 60, 90, and 120 seconds). Brain uptake was expressed as apparent brain distributional volume, V_{brain}, in the left hemisphere during perfusion (flow rate: 2.1 ml/min). Data are presented as mean ± S.E.M. (n = 4 mice).

Fig. 3. Brain uptake rate of SQV in the left hemisphere as a function of SQV concentration in perfusate. Line represents the fitting by a modified Michaelis-Menten equation ($J=K_d S-[J_{max}S/(K_m+S)]$), where J_{max} is the maximum uptake rate for saturable component, K_m is the Michaelis constant, K_d is the first-order constant for the non-saturable component and S is the concentration of SQV ($r^2 = 0.97$). Data are means ± S.E.M. (n = 4).

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Fig. 4. Apparent brain distributional volumes of SQV (◊) versus various concentrations of GF120918 which was included in the perfusates: Brain vascular volume was measured using [^{3}H] inulin (●). The mice were perfused for 60 s. Perfusion flow rate: 2.1 ml/ min. Data are means ± S.E.M. (n = 3 ~ 4).

Fig. 5. Apparent brain distributional volumes of SQV (◊) versus various concentrations of MK571 which was included in the perfusates: Brain vascular volume was measured using [^{3}H] inulin (●). The mice were perfused for 60 s. Perfusion flow rate: 2.1 ml/ min. Data are means ± S.E.M. (n = 3 ~ 4).

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