

# In Vivo Evaluation of P-Glycoprotein Modulation of 8 PET Radioligands Used Clinically

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P-glycoprotein (P-gp) regulates the ability of endogenous and exogenous compounds to cross the blood–brain barrier. We investigated whether PET tracers used clinically for studying brain function are affected by P-gp. **Methods:** Modulation of 8 radioligands by P-gp was assayed in mice by evaluating the effect of treatment with cyclosporine A (CsA) on uptake into the brain (assay 1) and the effect of treatment with a cold ligand of the corresponding radioligand on uptake of <sup>11</sup>C-verapamil, a representative radioligand for P-gp (assay 2). Brain-to-blood ratios were also examined as the other index to correct the delivery of radioligands. The radioligands investigated were <sup>11</sup>C-TMSX (adenosine A<sub>2A</sub> receptor), <sup>11</sup>C-MPDX (adenosine A<sub>1</sub> receptor), <sup>11</sup>C-PK11195 (peripheral benzodiazepine receptor), <sup>11</sup>C-flumazenil (central benzodiazepine receptor), <sup>11</sup>C-raclopride (dopamine D<sub>2</sub>-like receptor), <sup>11</sup>C-pyramine (histamine H<sub>1</sub> receptor), <sup>11</sup>C-PIB (amyloid plaque), and <sup>11</sup>C-donepezil (acetylcholine esterase). **Results:** In assay 1, CsA treatment increased both the uptake and the brain-to-blood ratio of <sup>11</sup>C-TMSX, <sup>11</sup>C-MPDX, <sup>11</sup>C-flumazenil, and <sup>11</sup>C-donepezil among the 8 radioligands. In assay 2, in which 4 cold ligands were examined, cold verapamil slightly increased the brain-to-blood ratio of <sup>11</sup>C-verapamil, but TMSX, MPDX, and MPPF did not increase either parameter. **Conclusion:** Assay 1 was suitable for evaluating the P-gp modulation of radioligands. Among the 8 radioligands investigated, <sup>11</sup>C-TMSX, <sup>11</sup>C-MPDX, <sup>11</sup>C-flumazenil, and <sup>11</sup>C-donepezil were modulated by P-gp.

**Key Words:** PET radioligand; P-glycoprotein; cyclosporine A; neuroreceptor

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**T**he permeability of endogenous and exogenous compounds across the blood–brain barrier is regulated by transporters such as P-glycoprotein (P-gp), multidrug-resistance-associated protein, and organic anion transporters. Furthermore, these transporters can also be expressed on the membrane of tumor cells. Chemotherapeutic treat-

ment of cancer patients is often unsuccessful because of the involvement of various mechanisms leading to multidrug resistance, in which P-gp is involved in effluxion of drugs. PET and SPECT can be used to investigate the functionality of these transporters in the human brain and in tumors in vivo (1–4). So far, several radioligands for PET and SPECT have been proposed (5–7), and <sup>11</sup>C-verapamil and <sup>99m</sup>Tc-sestamibi have been used in clinical studies (8–13). On the other hand, some radioligands for mapping neuroreceptors are also substrates for P-gp: <sup>11</sup>C-carazolol and <sup>18</sup>F-fluorocarazolol for  $\beta$ -adrenergic receptor (14) and <sup>18</sup>F-MPPF for serotonin 5-HT<sub>1A</sub> receptor (15). These radioligands showed a relatively fast clearance of radioactivity from the brain of rodents; however, the uptake was remarkably enhanced on treatment with cyclosporine A (CsA), a so-called modulator of P-gp (Table 1). These phenomena result from inhibition of radioligand efflux from the endothelial membrane into plasma. When we compared the in vivo brain kinetics of 2  $\sigma_1$ -receptor radioligands, <sup>3</sup>H-pentazocine and <sup>11</sup>C-SA4503, in mice, the former showed a much faster clearance and a much lower specific binding than the latter despite similar affinities for  $\sigma_1$ -receptors (16). The different kinetics were explained by the finding that <sup>3</sup>H-pentazocine was modulated by P-gp but that <sup>11</sup>C-SA4503 was not. In the case of <sup>11</sup>C-GR218231, a dopamine D<sub>3</sub> receptor antagonist, brain uptake was low because of P-gp, and no receptor-specific binding was found (17). Therefore, it is important to the development of new radioligands for PET or SPECT whether they are modulated by P-gp. It is also of interest whether the radiotracers presently used in clinical studies are modulated by P-gp.

So far, the modulation of radioligands by P-gp in vivo has been evaluated in rodents treated with CsA. In this study, we investigated 2 assays for evaluating P-gp modulation of radioligands in vivo. Assay 1 was a standard method previously used (8,14–17). Mice were intravenously treated with CsA, and the brain uptake of a radioligand was examined to see whether it could be enhanced. In assay 2, we examined whether uptake of <sup>11</sup>C-verapamil, a representative probe for P-gp, was affected by cold ligand. When cold ligands directly modulate P-gp or compete with <sup>11</sup>C-verapamil in drug efflux by P-gp, uptake of <sup>11</sup>C-verapamil will be enhanced. If assay 2

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**TABLE 1**  
Effects of CsA on Brain Uptake of Radioactivity After Injection of Each Radioligand into Mice

Radioligand	Brain uptake of radioligand			Brain-to-blood ratio		
	Control (%ID/g)	Treatment (%ID/g)	Ratio	Control	Treatment	Ratio
In this study						
<sup>11</sup> C-Verapamil	0.40 ± 0.04	4.44 ± 0.52*	11.05	0.50 ± 0.08	4.35 ± 0.66*	8.76
<sup>11</sup> C-TMSX	1.92 ± 0.17 <sup>‡</sup>	3.24 ± 0.38*	1.69	1.29 ± 0.13	2.04 ± 0.32*	1.59
<sup>11</sup> C-MPDX	1.87 ± 0.26	3.67 ± 0.84 <sup>†</sup>	1.96	0.79 ± 0.11	1.47 ± 0.43 <sup>†</sup>	1.86
<sup>11</sup> C-PK11195	1.05 ± 0.05	1.71 ± 0.62	1.33	2.07 ± 0.36	1.56 ± 0.92	0.86
<sup>11</sup> C-Flumazenil	2.25 ± 0.27	7.54 ± 1.09*	3.35	2.94 ± 0.53	4.62 ± 0.65 <sup>†</sup>	1.57
<sup>11</sup> C-Raclopride	1.16 ± 0.17 <sup>¶</sup>	1.70 ± 0.28 <sup>†</sup>	1.46	1.50 ± 0.20	1.16 ± 0.36	0.77
<sup>11</sup> C-Pyridamine	2.71 ± 0.18	4.61 ± 0.42*	1.70	7.07 ± 0.78	3.21 ± 0.30*	0.46
<sup>11</sup> C-PIB	0.56 ± 0.07	0.75 ± 0.11	1.34	0.56 ± 0.09	0.34 ± 0.06	0.61
<sup>11</sup> C-Donepezil	1.91 ± 0.20	6.13 ± 1.07*	3.21	4.60 ± 0.44	10.93 ± 2.34*	2.38
Previously reported <sup>§</sup>						
<sup>11</sup> C-Carazolol (14)	0.12 ± 0.03	0.62 ± 0.05	5.17	1.50	2.50	1.67
<sup>18</sup> F-Fluorocarazolol (14)	0.77 ± 0.19	1.82 ± 0.22	2.34	2.75	5.87	2.13
<sup>18</sup> F-MPPF (15)	0.22	1.15	5.23	1.47	2.41	1.64
<sup>11</sup> C-SA4503 (16)	3.96 ± 0.20	3.84 ± 0.55	0.99	4.19 ± 0.73	4.33 ± 0.80	1.03
<sup>11</sup> C-GR218231 (17)	0.21 ± 0.05	2.85 ± 0.56	13.6	0.22	2.50	11.4

\* $P < 0.001$  (Student  $t$  test, compared with control).

<sup>†</sup> $P < 0.01$  (Student  $t$  test, compared with control).

<sup>‡</sup>%ID/g in striatum rich in corresponding receptors was  $4.99 \pm 0.46$ .

<sup>¶</sup>%ID/g in striatum rich in corresponding receptors was  $5.30 \pm 0.72$ .

<sup>§</sup>Data are quoted or recalculated from the original reports, where assay 1 was performed on rats and levels of radioactivity in frontal cortex (14), hippocampus (15), whole brain (16), or neocortex (17) and plasma, instead of blood, were expressed as standardized uptake value: (radioactivity of tissue/gram of tissue)/(total injected radioactivity/gram of whole-body weight). Significant modulation by CsA was reported for 4 radioligands but not <sup>11</sup>C-SA4503.

Data are mean  $\pm$  SD ( $n = 4-6$ ).

could be used for the evaluation of candidate PET radioligands, they could be screened in vivo before radiolabeling.

For the assays, we selected 8 radioligands for PET that have been used clinically at the Tokyo Metropolitan Institute of Gerontology (TMIG) or Tohoku University: <sup>11</sup>C-TMSX, <sup>11</sup>C-MPDX, <sup>11</sup>C-PK11195, <sup>11</sup>C-flumazenil, <sup>11</sup>C-raclopride, <sup>11</sup>C-pyridamine, <sup>11</sup>C-PIB, and <sup>11</sup>C-donepezil (Fig. 1). These 8 radioligands were compared with <sup>11</sup>C-verapamil and other tracers previously reported. <sup>11</sup>C-TMSX and <sup>11</sup>C-MPDX are xanthine-type compounds for mapping adenosine A<sub>2A</sub> and A<sub>1</sub> receptors, respectively, and were originally developed at the TMIG (18–20). <sup>11</sup>C-Pyridamine has been used for the imaging of histamine H<sub>1</sub> receptors in at least 2 PET centers, including Tohoku University (21,22). <sup>11</sup>C-Donepezil PET is being tested clinically for imaging acetylcholine esterase at Tohoku University (23). The labeled position of the <sup>11</sup>C-donepezil used clinically differs from that of the <sup>11</sup>C-donepezil prepared by De Vos et al. (24). <sup>11</sup>C-PIB has been developed for imaging amyloid plaque (25) and used at many PET centers, and the other 3 tracers, <sup>11</sup>C-PK11195, <sup>11</sup>C-flumazenil, and <sup>11</sup>C-raclopride, have been widely used. All of the selected radioligands show relatively fast brain kinetics.

## MATERIALS AND METHODS

### General

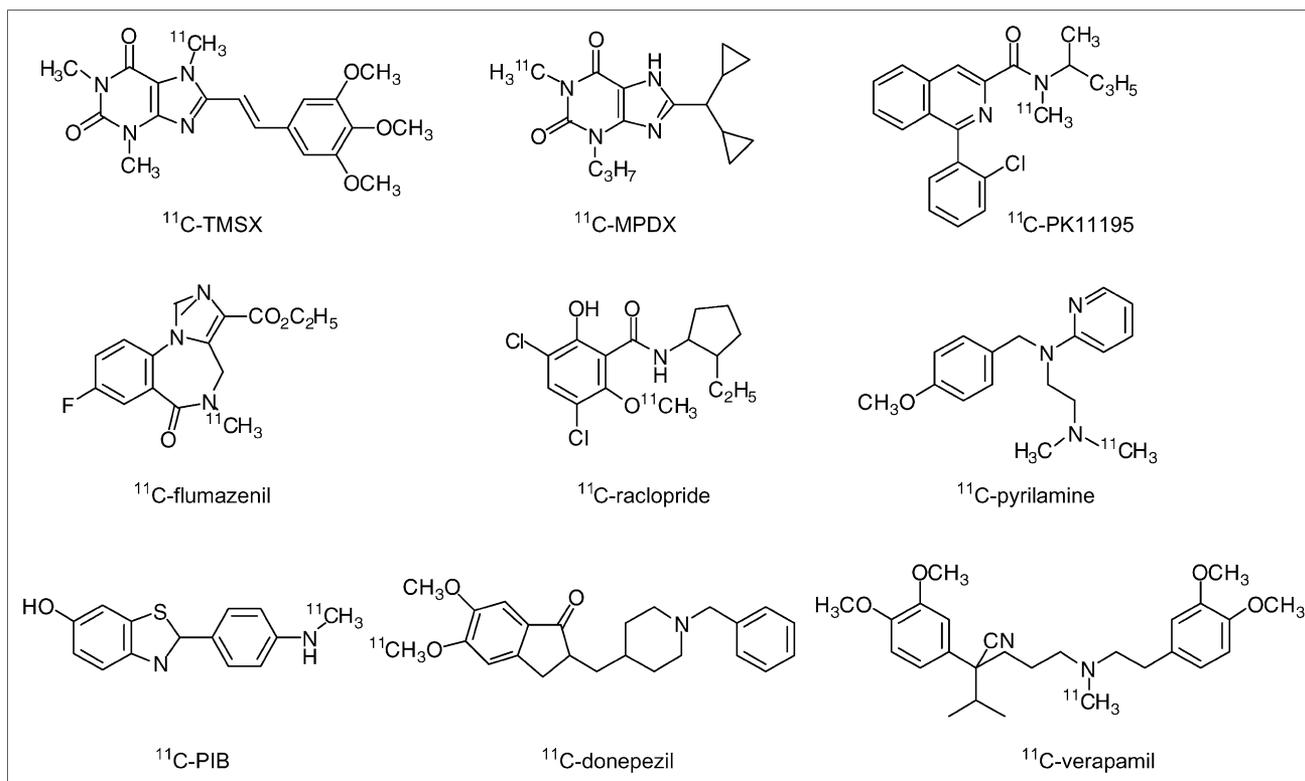
CsA (Sandimmun) was purchased from Novartis Pharma; verapamil hydrochloride, from Sigma-Aldrich; MPPF dihydro-

chloride, from Research Biochemicals International; and dimethyl sulfoxide (DMSO), from Wako Pure Chemical Industries. <sup>11</sup>C-TMSX (26), <sup>11</sup>C-MPDX (27), <sup>11</sup>C-PK11195 (28), <sup>11</sup>C-flumazenil (29), <sup>11</sup>C-raclopride (30), <sup>11</sup>C-pyridamine (31), <sup>11</sup>C-PIB (32), <sup>11</sup>C-donepezil (23), and <sup>11</sup>C-verapamil (33) were prepared at the TMIG as described previously (23,26–33).

Male ddY mice were purchased from Tokyo Laboratory Animals. All experiments with animals were approved by the Animal Care and Use Committee of the TMIG.

### Assay 1

The effects of CsA on uptake of radioactivity into the brain were investigated for all 8 radioligands—<sup>11</sup>C-TMSX, <sup>11</sup>C-MPDX, <sup>11</sup>C-PK11195, <sup>11</sup>C-flumazenil, <sup>11</sup>C-raclopride, <sup>11</sup>C-pyridamine, <sup>11</sup>C-PIB, and <sup>11</sup>C-donepezil—and for <sup>11</sup>C-verapamil, a standard radioligand for P-gp, as a reference. Assay 1 is a standard method (8,14–17), but the time course of the effect of CsA has not been reported. Therefore, first, the time course of the effect of CsA was investigated using <sup>11</sup>C-verapamil to determine the optimal period before injection of the tracer. The mice received an intravenous injection of CsA, which was dissolved at a concentration of 10 mg/mL in physiologic saline. The dose of CsA was 50 mg/kg, and the volume of the mixture was 5 mL/kg. Thirty, 60, 120, and 240 min later, <sup>11</sup>C-verapamil was injected via the tail into 4 groups of mice. Control mice were treated with physiologic saline at 5 mL/kg and then given <sup>11</sup>C-verapamil 30 min later. They were killed by cervical dislocation 30 min after the injection. The 30-min period was chosen because uptake of <sup>11</sup>C-verapamil into the brain remained constant for the first 60 min after injection (6). Blood was collected



**FIGURE 1.** Chemical structures of radioligands investigated.

by heart puncture using a syringe, and the brain was harvested. The samples were measured for  $^{11}\text{C}$ -radioactivity with an auto- $\gamma$ -counter and weighed. Tissue uptake of radioactivity was expressed as a percentage of the injected dose per gram of tissue (%ID/g).

In the same way, each of the other 8 radioligands was intravenously injected into the mice 30 min after intravenous injection of CsA. Control mice were given physiologic saline. Fifteen minutes after injection of each radioligand, the mice were killed by cervical dislocation and blood and brain were obtained as described above. The 15-min period was chosen because all 8 radioligands showed a similar pattern of clearance, that is, the highest uptake was observed within 15 min after injection. In the case of  $^{11}\text{C}$ -TMSX, the target tissue (rich in adenosine  $A_{2A}$  receptors) is the striatum (18,26), as it is in the case of  $^{11}\text{C}$ -raclopride. Peripherally,  $^{11}\text{C}$ -TMSX specifically bound to the adenosine  $A_{2A}$  receptors in the heart and muscle (34). Therefore, the heart and muscle were also obtained. The brains of the  $^{11}\text{C}$ -TMSX- or  $^{11}\text{C}$ -raclopride-injected mice were further divided into the cerebral cortex, striatum, cerebellum, and remainder, and those of the  $^{11}\text{C}$ -MPDX-injected mice, into the cerebral cortex, hippocampus, cerebellum, and remainder. Uptake of  $^{11}\text{C}$ -MPDX was relatively high in the hippocampus (27,35). Tissue uptake of radioactivity was expressed as %ID/g.

## Assay 2

The effects of cold ligands on uptake of  $^{11}\text{C}$ -verapamil were investigated. We selected verapamil, TMSX, and MPDX as cold ligands and MPPF as a reference ligand that was modulated by P-gp (15). Cold verapamil was dissolved at 0.2 or 1.0 mg/mL in physiologic saline, and TMSX and the other 2 ligands were

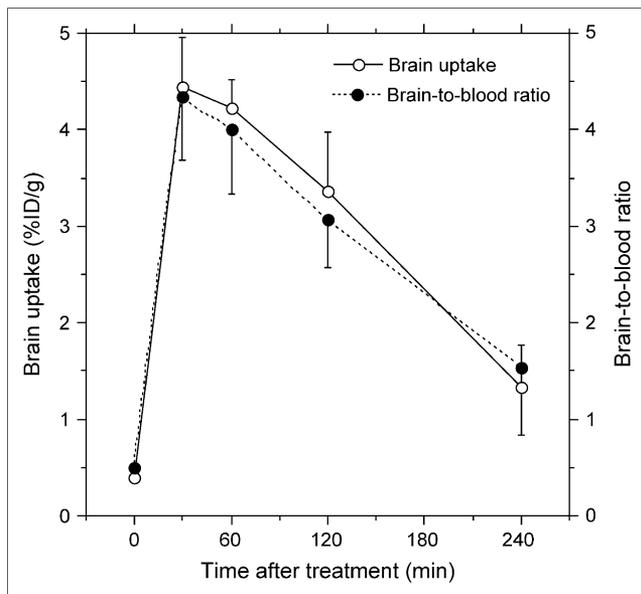
dissolved at 0.1 and 0.2 mg/mL, respectively, in a mixture of physiologic saline and DMSO (1/1, v/v). The dose of verapamil was 1 or 5 mg/kg, and the doses of TMSX and the other 2 ligands were 0.5 and 1 mg/kg. The volume injected was 5 mL/kg for all ligands.

The mice received an intravenous injection of each of the 4 cold ligands. Control mice for the verapamil group received physiologic saline, and control mice for the other groups received a mixture of physiologic saline and DMSO (1/1, v/v) at a concentration of 5 mL/kg. Thirty or 10 min after injection of the verapamil and each of the other ligands,  $^{11}\text{C}$ -verapamil was intravenously injected. The 30-min period was selected for verapamil because uptake of  $^{11}\text{C}$ -verapamil remained constant for the first 60 min (6), and the 10-min period was selected for the others because  $^{11}\text{C}$ -TMSX,  $^{11}\text{C}$ -MPDX, and  $^{18}\text{F}$ -MPPF rapidly washed out from the brain after the injection (15,26,27). The mice were killed by cervical dislocation 30 min after injection, and blood and brain were obtained. In the case of TMSX, the heart and muscle were also obtained. The brains of TMSX-pretreated mice were divided into the cerebral cortex, striatum, cerebellum, and remainder, and those of the MPDX- or MPPF-pretreated mice, into the cerebral cortex, hippocampus, cerebellum, and remainder. Tissue uptake of radioactivity was expressed as %ID/g.

## RESULTS

### Assay 1

To determine the optimal time after treatment with CsA, the time course of the effects of CsA on uptake of  $^{11}\text{C}$ -verapamil was investigated 4 h after the treatment. Figure 2



**FIGURE 2.** Time course of effects of CsA on brain uptake of  $^{11}\text{C}$ -verapamil and brain-to-blood ratios. Data are mean  $\pm$  SD ( $n = 5$ ).

shows that the largest effect was observed at 30 min (1,105% of the control) and that the effects gradually decreased (293% of the control at 4 h). The effects on the ratio of brain to blood were slightly less than those on uptake into the brain.

The effects of treatment with CsA on the 9 radioligands are summarized in Table 1. The effects on another 5 radioligands previously reported are also included as references. Uptake was significantly enhanced for 6 of the 8 radioligands, in addition to  $^{11}\text{C}$ -verapamil. However, the brain-to-blood ratio for  $^{11}\text{C}$ -raclopride was not affected, and that for  $^{11}\text{C}$ -pyrilamine was significantly decreased. Thus, treatment with CsA significantly enhanced both uptake into the brain and the brain-to-blood ratio for  $^{11}\text{C}$ -TMSX,  $^{11}\text{C}$ -MPDX,  $^{11}\text{C}$ -flumazenil, and  $^{11}\text{C}$ -donepezil.

Figure 3A shows the effects of CsA on  $^{11}\text{C}$ -TMSX radioactivity levels in the 3 regions of the brain and in heart, muscle, and blood. In the brain, uptake of  $^{11}\text{C}$ -TMSX was enhanced to a similar extent both in the adenosine  $\text{A}_{2\text{A}}$  receptor-rich striatum and in the adenosine  $\text{A}_{2\text{A}}$  receptor-poor cerebral cortex and cerebellum but not in the heart and

muscle. In the striatum, the receptor-specific uptake (%ID/g) determined by subtracting cerebellar uptake from striatal uptake significantly increased:  $3.21 \pm 0.33$  versus  $5.89 \pm 0.94$  ( $P < 0.001$ ), whereas the target-to-nontarget ratio (striatum-to-cerebellum ratio) was not affected:  $2.81 \pm 0.17$  versus  $3.03 \pm 0.23$ . Similar effects of CsA on the levels of radioactivity of  $^{11}\text{C}$ -MPDX or  $^{11}\text{C}$ -raclopride were also found in all 3 regions of the brain (data not shown).

## Assay 2

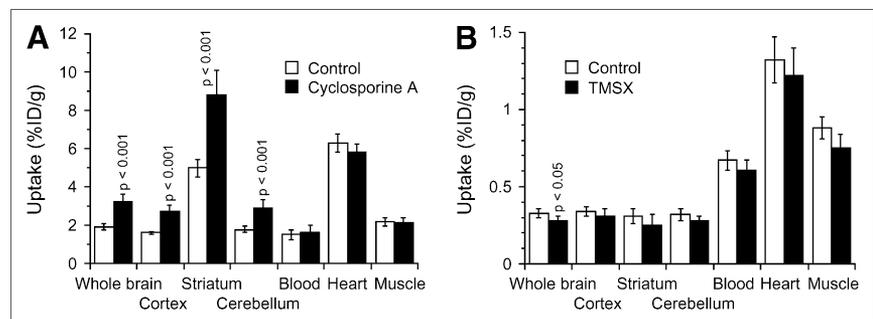
Table 2 summarizes the effects of 4 cold ligands (verapamil, TMSX, MPDX, and MPPF) on uptake into the brain and the brain-to-blood ratio for  $^{11}\text{C}$ -verapamil. Cold verapamil at a dose of 5 mg/kg slightly increased the brain-to-blood ratio but not uptake. Uptake was slightly decreased by cold TMSX, but the brain-to-blood ratio was not affected. MPDX and MPPF had no effect on either parameter.

Figure 3B shows the effects of cold TMSX on the levels of radioactivity of  $^{11}\text{C}$ -verapamil in the 3 regions of the brain and in heart, muscle, and blood. There was a tendency for levels to decrease slightly in all tissues investigated, but the decrease was not significant. The other 2 cold ligands had no effect on the levels of radioactivity of  $^{11}\text{C}$ -verapamil in the 3 regions of the brain (data not shown).

## DISCUSSION

Because of increased evidence that transporters have important roles in the movement of endogenous and exogenous compounds across the blood-brain barrier or the plasma membrane in tumors and other tissues, PET and SPECT have been used to investigate the functionality of transporters (1-4). However, it has also been reported that several radioligands for mapping neuroreceptors by PET are affected by P-gp (14,15), although many PET radioligands used clinically have not been evaluated for modulation by P-gp. In the present study, we investigated the possibility of modulation for 8 radioligands. All of the selected radioligands are in clinical use and show relatively fast brain kinetics, that is, an apparent equilibrium state is reached by 60 min. We excluded radioligands showing accumulation patterns, such as  $^{11}\text{C}$ -SCH 23390 (dopamine  $\text{D}_1$  receptor),  $^{11}\text{C}$ -*N*-methylspiperone (dopamine  $\text{D}_2$ -like receptor),  $^{11}\text{C}$ - $\beta$ -CFT (dopamine transporter),  $^{11}\text{C}$ -doxepin

**FIGURE 3.** Uptake of radiotracers in brain, heart, and muscle in the 2 assays for evaluating P-gp modulation. (A) Assay 1: effect of treatment with CsA on uptake of  $^{11}\text{C}$ -TMSX. (B) Assay 2: effect of treatment with cold TMSX on uptake of  $^{11}\text{C}$ -verapamil. Data are mean  $\pm$  SD ( $n = 5-6$ ). Student *t* test was performed for comparison with control data.



**TABLE 2**  
Effects of Cold Ligands on Brain Uptake of Radioactivity 30 Minutes After Injection of  $^{11}\text{C}$ -Verapamil into Mice

Cold ligand	Dose (mg/kg)	Brain uptake of $^{11}\text{C}$ -verapamil			Brain-to-blood ratio		
		Control (%ID/g)	Treatment (%ID/g)	Ratio	Control	Treatment	Ratio
Verapamil	1	0.38 ± 0.05	0.36 ± 0.06	0.97	0.50 ± 0.05	0.53 ± 0.06	1.07
	5	0.38 ± 0.05	0.50 ± 0.14	1.32	0.50 ± 0.05	0.65 ± 0.16*	1.31
TMSX	0.5	0.33 ± 0.03	0.28 ± 0.03*	0.85	0.49 ± 0.06	0.46 ± 0.06	0.94
MPDX	1	0.36 ± 0.04	0.35 ± 0.02	0.97	0.53 ± 0.04	0.53 ± 0.05	1.00
MPPF	1	0.36 ± 0.04	0.35 ± 0.06	0.97	0.53 ± 0.04	0.54 ± 0.10	1.02

\* $P < 0.05$  (Student  $t$  test, compared with control).

Data are mean ± SD ( $n = 5-6$ ).

(histamine<sub>1</sub> receptor), and  $^{11}\text{C}$ -deprenyl (monoamine oxidase-B inhibitor). Two assays were used to detect modulation by P-gp.

Assay 1 was a standard method (8,14–17). Because uptake of the tracer could be partially affected by treatment with CsA, modulation was evaluated using 2 indices, brain uptake and brain-to-blood ratio. The level of radioactivity in blood at a single time point does not reflect the area under the curve of the tracer in plasma; however, the brain-to-blood ratio can be used as an index as demonstrated for  $^{11}\text{C}$ -verapamil (8). In the present study, we also confirmed that the 2 indices of  $^{11}\text{C}$ -verapamil were affected to a similar extent (Fig. 2). We found that treatment with CsA significantly increased both indices for 4 radioligands:  $^{11}\text{C}$ -TMSX,  $^{11}\text{C}$ -MPDX,  $^{11}\text{C}$ -flumazenil, and  $^{11}\text{C}$ -donepezil. The enhancement was much less than that observed for  $^{11}\text{C}$ -verapamil or the other probe for P-gp,  $^{11}\text{C}$ -GR218231 (17), but was comparable to that induced by  $^{11}\text{C}$ -carazolol and  $^{18}\text{F}$ -fluorocarazolol (14) or  $^{18}\text{F}$ -MPPF (15) (Table 1). The increased uptake of  $^{11}\text{C}$ -TMSX or  $^{11}\text{C}$ -MPDX was observed to a similar degree in 3 brain regions independently of the densities of the corresponding receptors (Fig. 3A), as observed for  $^{11}\text{C}$ -carazolol,  $^{18}\text{F}$ -fluorocarazolol, and  $^{18}\text{F}$ -MPPF (15,16). It is known that  $^{11}\text{C}$ -TMSX binds peripherally to adenosine A<sub>2A</sub> receptors in the heart and muscle (34); however, uptake in these tissues was not influenced by treatment with CsA (Fig. 3A). Therefore, we concluded that  $^{11}\text{C}$ -TMSX,  $^{11}\text{C}$ -MPDX,  $^{11}\text{C}$ -flumazenil, and  $^{11}\text{C}$ -donepezil were modulated by P-gp.

Although assay 1 was convenient as the first screening, the limitations of that assay in the present study should be noted. For assay 1, we carefully selected a single time point for each ligand based on the previous reports; however, CsA perturbations may alter ligand kinetics in the brain and peripheral metabolism. The brain-to-blood ratio at a single time point is not an adequate index of ligand delivery. Therefore, multiple-time-point analyses or dynamic PET measurement is preferable for elaboration of each ligand (8).

In assay 2, we studied verapamil, TMSX, MPDX, and MPPF. Only verapamil at a dose of 5 mg/kg increased (slightly) the brain-to-blood ratio of  $^{11}\text{C}$ -verapamil, by 1.31-fold; however, a 1.32-fold uptake increase in the brain

was not significant (Table 2). The findings in this assay may be due to the low doses of cold ligands used. For instance, we used 1 or 5 mg (2 or 10  $\mu\text{mol}$ ) of cold verapamil per kilogram of body weight to modulate P-gp, but the in vitro assay using cell cultures described below used 20  $\mu\text{mol}$  of verapamil per liter to inhibit transport of substrates for P-gp (36). In assay 1, the amount of CsA used to modulate P-gp was 50 mg/kg. The modulation occurred dose-dependently, and doses of less than 10 mg/kg were not effective (4,17, 37). However, because of the toxicity of cold ligands, their doses could not be increased. The median lethal dose of verapamil in mice is 8 mg/kg intravenously (38), and a preliminary experiment showed that DMSO solutions containing larger doses of the other ligands were not acceptable (data not shown). Another reason for the findings may be the modulation period between injection of each cold ligand and injection of each tracer or the interval allowed for brain uptake after injection of each tracer. These intervals were determined on the basis of the brain kinetics of radiolabeled ligands as described in the “Materials and Methods” but may not have been optimal for the assays. Optimization of this assay for each ligand and further examination of other ligands with this assay may be required, but we did not perform further studies because a small signal for possible modulation of P-gp was observed only for verapamil. Three other ligands enhanced neither the brain uptake of  $^{11}\text{C}$ -verapamil nor the brain-to-blood ratio at doses appropriate for blockade of many neuroreceptors in vivo, suggesting that cold ligands did not modulate P-gp at the doses used or that efflux of  $^{11}\text{C}$ -verapamil by P-gp was not saturated with cold ligands. We also confirmed that cold verapamil at doses of 1 and 5 mg/kg did not affect brain uptake of  $^{11}\text{C}$ -TMSX and  $^{11}\text{C}$ -MPDX (data not shown). If detailed optimization is essential for each test ligand for which the brain kinetics in vivo are unknown, assay 2 has no practical benefit. Therefore, we concluded that modulation of radioligands by P-gp could not be evaluated in vivo by assay 2.

Transcellular transport studies for determining whether compounds are substrates for the P-gp efflux pump are usually performed using P-gp-overexpressing cell lines (36,37). Before labeling candidate compounds for PET, screening of these candidates with this assay is preferable

for the development of PET radioligands; however, *ex vivo* biodistribution studies using labeled compounds (assay 1 in the present study) may be more sensitive for detecting P-gp modulation. Bart et al. found that CsA treatment resulted in a 5-fold increase in  $^{11}\text{C}$ -carvedilol in rat brain and a 3-fold increase in a human small-cell lung carcinoma cell subline overexpressing P-gp (37). For the assay with *ex vivo* biodistribution studies, the use of P-gp knockout mice is another choice (6,14,15,37); however, assay 1 using conventional rodents and CsA is much more convenient.

When PET radioligands are modulated by P-gp, how are the PET data influenced? As reported previously (14,15) and also shown in Figure 3A, CsA treatment enhanced uptake of the radioligand in all regions of the brain, including targeted and nontargeted tissues. Thus, the receptor-specific uptake determined by subtracting uptake in nontargeted tissues from uptake in targeted tissues significantly increased, whereas PET signals revealing the specific binding ratio of each ligand to its corresponding receptor, that is, target-to-nontarget ratio, were not affected. From this point of view, distribution of the receptors represented by the radioligands may be unaffected regardless of whether they are modulated by P-gp. However, because the specific signals become much lower than those of radioligands not regulated by P-gp, subtle alterations in the brain may be missed. Furthermore, Kortekaas et al., using  $^{11}\text{C}$ -verapamil PET, recently found that P-gp function is reduced in the midbrain of patients with Parkinson's disease and suggested that this dysfunction is part of the pathogenesis of Parkinson's disease (9). Therefore, if a PET radioligand is modulated by P-gp, studies using that radioligand should be performed carefully and with consideration of the extent of P-gp modulation, especially in patients treated with P-gp modulators such as CsA or with P-gp substrates (3). One should keep in mind that other transporters, such as multidrug-resistance-associated protein and organic anion transporters, may also modulate the ability of radioligands to cross the blood-brain barrier and the plasma membranes of tumors and other tissues.

## CONCLUSION

Modulation by P-gp of 8 PET radioligands was assayed in mice. CsA treatment increased both the brain uptake and the brain-to-blood ratio of  $^{11}\text{C}$ -TMSX,  $^{11}\text{C}$ -MPDX,  $^{11}\text{C}$ -flumazenil, and  $^{11}\text{C}$ -donepezil. Treatment with the cold ligand of the corresponding radioligand was not suitable for determining P-gp modulation of  $^{11}\text{C}$ -verapamil uptake by the brain. We concluded that of the radioligands investigated,  $^{11}\text{C}$ -TMSX,  $^{11}\text{C}$ -MPDX,  $^{11}\text{C}$ -flumazenil, and  $^{11}\text{C}$ -donepezil were modulated by P-gp.

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