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In vitro characterization of radioiodinated (+)-2-[4-(4-iodophenyl) piperidino]cyclohexanol [(+)-pIV] as a sigma-1 receptor ligand

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Abstract—We investigated the binding characteristics of a (+)-enantiomer of radioiodinated 2-[4-(4-iodophenyl)piperidino]cyclohexanol [(+)-[^{125}I]pIV], radioiodinated at the *para*-position of the 4-phenylpiperidine moiety, to sigma receptors (σ-1, σ-2) and to vesicular acetylcholine transporters (VAChT) in membranes of the rat brain and liver. In competitive inhibition studies, (+)-pIV (K_i = 1.30 nM) had more than 10 times higher affinity to the sigma-1 (σ-1) receptor than (+)-pentazocine (K_i = 19.9 nM) or haloperidol (K_i = 13.5 nM) known as sigma ligands. Also, the binding affinity of (+)-pIV for the σ-1 receptor (K_i = 1.30 nM), was about 16 times higher than the sigma-2 (σ-2) receptor (K_i = 20.4 nM). (+)-pIV (K_i = 1260 nM) had a much lower affinity for VAChT than (−)-vesamicol (K_i = 13.0 nM) or (−)-pIV (K_i = 412 nM). (+)-[^{125}I]pIV had low affinity for the dopamine, serotonin, adrenaline, and acetylcholine receptors. Furthermore, in a saturation binding study, (+)-[^{125}I]pIV exhibited a K_d of 6.96 nM with a B_{max} of 799 fmol/mg of protein. These results showed that (+)-pIV binds to the σ-1 receptor with greater affinity than sigma receptor ligands such as (+)-pentazocine or haloperidol, and that radioiodinated (+)-pIV is suitable as radiotracer for σ-1 receptor studies in vitro.

1. Introduction

It is known that sigma receptors are distributed at a comparatively high density to the limbic system and cortex that have a close relationship with schizophrenia.[1–4] Sigma receptors specifically change in the limbic system and a cortex in the brain after the death of a patient with schizophrenia.[5–7] Furthermore, it has been reported that sigma receptors promote release of acetylcholine in the cerebral cortex frontal lobe and hippocampus[8] and that learning/memory impairment is improved by sigma receptors.[9–12] Sigma receptors are thought to be closely related to learning/memory mechanism. Thus, it is useful to develop various sigma receptor ligands with which to investigate the central nervous system (CNS).

Sigma receptors have been categorized into at least two subtypes, termed as sigma-1(σ-1) and sigma-2 (σ-2).[13] The σ-1 receptor exhibits a high affinity for (+)-benzomorphans, whereas the σ-2 receptor displays a low affinity for (+)-benzomorphans.[14] Most known σ receptor ligands are either selective for the σ-1 receptor or are relatively nonselective, such as haloperidol and 1,3-di-tolylguanidine (DTG). The benzomorphans, such as N-allylnormetazocine ((+)-SKF 10047) and (+)-pentazocine are among the compounds, which show the highest σ-1/σ-2 selectivity; however, these compounds also bind to other neuroreceptors.[15]

In previous studies, we reported that the binding affinity for acetylcholine transporter (VAChT) and sigma receptors (σ-1, σ-2) of vesamicol varies with the position of iodine introduced into the 4-phenylpiperidine moiety of vesamicol.[16,17] In this study, we synthesized the (+)-enantiomer of radioiodinated 2-[4-(4-iodophenyl)piperidino]cyclohexanol [(+)-[^{125}I]pIV] and investigated the in vitro binding characteristics of (+)-[^{125}I]pIV to σ-1 receptor in the rat brain to evaluate the potential usefulness of (+)-[^{125}I]pIV as radiotracer for σ-1 receptor studies in vitro.

2. Material and methods

(−)-[^{3}H]vesamicol (1.30 TBq/mmol), [^{3}H]1,3-di-tolylguanidine ([^{3}H]DTG) (1.1 TBq/mmol), [^{3}H]pentazocine (1.0 TBq/mmol), and [^{125}I]sodium iodide (644 GBq/mg) were purchased.

Keywords: (+)-pIV; Vesamicol; Sigma receptors; Radioligand.
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2.1. Synthesis of (+)-*p*-iodovesamicol ((+)-pIV)

The (+)-enantiomer of (+)-pIV was prepared using a method described previously,[16] but using the corresponding enantiomer of vesamicol (Fig. 1). Briefly, racemic vesamicol was synthesized from the 4-phenylpridine via a two-step reaction. The (+)-enantiomer of vesamicol was provided from racemic vesamicol by recrystallizing the diastereoisomeric salts using (+)-di-*p*-toluoyl-D-tartaric acid monohydrate. (+)-pIV was synthesized from the (+)-enantiomer of vesamicol via a three-step reaction.

2.2. Characteristics of (+)-2-[4-(4-iodophenyl)piperidino]cyclohexanol[(+)-pIV]

Melting point: 162–164 °C. NMR ($CDCl_3$): δ 1.19–1.25 (4H, m), 1.60–1.85 (8H, m), 2.12–2.15 (1H, m), 2.20–2.25 (2H, m), 2.41–2.45 (1H, m), 2.71–2.75 (2H, m) 2.92–2.95 (1H, m), 3.37–3.41 (1H, m), 6.97 (2H, d, J = 8.30 Hz), 7.61 (2H, d, J = 8.30 Hz). Mass spectrum (m/e): 385 $[M]^+$. Elemental analysis $C_{17}H_{24}NOI$; theoretical values C: 53.00, H: 6.28, N: 3.64; experimental values C: 53.22, H: 6.44, N: 3.70. Specific rotation: $[\alpha]_D^{23} = -33.9$, (c 0.555, $CDCl_3$).

2.3. Radiolabeling

(+)-pIV was radioiodinated by solid-phase exchange with [^{125}I]NaI in the presence of ammonium sulfate as previously reported.[18]

2.4. Tissue preparation

Animal experiments were carried out in compliance with the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi Campus of Kanazawa University. Rat brain membranes and rat liver membranes were prepared from rat brains without a cerebellum and liver from male Sprague-Dawley rats (250–300 g) each using a method described previously.[17]

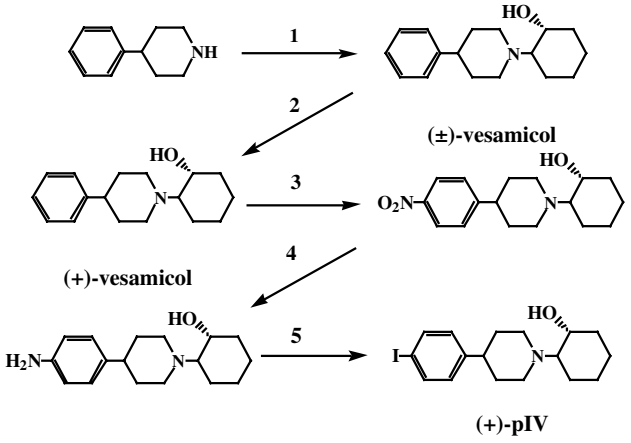

Figure 1. Synthesis of (+)-pIV. 1. Cyclohexene oxide; 2. (+)-di-*p*-toluoyl-D-tartaric acid; 3. HNO_3, H_2SO_4; 4. Fe, HCL; 5. $NaNO_2$, HCL, KI.

2.5. In vitro competitive binding study

2.5.1. VAChT binding. The assays were performed using a method reported previously[17] except that 514–717 μg protein of rat cerebral membranes were used. Briefly, 10 nM (−)-[^{3}H]vesamicol was used as a radioligand. Various concentrations of (+)-pIV, enantiomers of vesamicol or sigma ligands (from 10^{-10} to 10^{-5} M) were used as subject compounds. The mixture was incubated for 60 min at 37 °C in the presence of 100 nM haloperidol to mask sigma receptors.

2.5.2. σ-1 receptor binding. Rat cerebral membranes (514–717 μg protein) were incubated in triplicate with 3 nM (+)-[^{3}H]pentazocine and various concentrations of (+)-pIV, enantiomers of vesamicol or sigma ligands (from 10^{-10} to 10^{-5} M) in 0.5 mL of 50 mM Tris–HCl (pH 7.8) for 90 min at 37 °C. Nonspecific binding was determined in the presence of 10 μM (+)-pentazocine. The incubated samples were treated in the same manner as described for the VAChT binding assays.

2.5.3. σ-2 receptor binding. Rat liver membranes (150–300 μg protein) were incubated in triplicate with 3 nM [^{3}H]DTG and various concentrations of (+)-pIV, enantiomers of vesamicol or sigma ligands (from 10^{-10} to 10^{-5} M) in 0.5 mL of 50 mM Tris–HCl (pH 7.8) for 90 min at 37 °C in the presence of 1 μM (+)-pentazocine to mask σ-1 sites. Nonspecific binding was determined in the presence of 10 μM DTG and 1 μM (+)-pentazocine. The incubated samples were treated in the same manner as described for VAChT binding assays.

2.6. Saturation binding study

Rat cerebral membranes (514–717 μg protein) were incubated in triplicate with various concentrations of (+)-[^{125}I]pIV (from 1 to 60 nM) at 37 °C for 90 min. The incubated samples were quickly diluted, passed through glass-fiber filters (Whatman GF/B) and washed twice with an ice-cold buffer. The level of bound (+)-[^{125}I]pIV retained on the filter was measured in a gamma scintillation counter (Aloka, ARC-600). Nonspecific binding was determined in the presence of 10 μM of unlabeled (+)-pIV. A saturation binding study in the presence of 1 μM pentazocine was also done to mask the σ-1 receptor.

2.7. Inhibition of (+)-[^{125}I]pIV binding to rat cerebral membranes

Rat cerebral membranes (514–717 μg protein) were incubated in triplicate with 5 nM (+)-[^{125}I]pIV and various concentrations of (+)-pIV or reference compounds (from 10^{-10} to 10^{-5} M) in 0.5 mL of 50 mM Tris–HCl (pH 7.8) for 90 min at 37 °C. The incubated samples were treated in the same manner as described for the saturation binding assay.

2.7.1. Data analysis. K_i values in inhibition studies and K_d and B_{max} values in saturation binding studies were calculated with the 'Graphpad Prism' computer program (GraphPad Software, Inc. San Diego, USA).

Table 1. Affinities (nM) of (+)-pIV and reference compounds for VAChT and sigma receptors (σ-1, σ-2)

	VAChT	Sigma receptors	
		σ-1	σ-2
	K_i (nM)[a]	K_i (nM)[b]	K_i (nM)[c]
(+)-pIV	1260 ± 259	1.30 ± 0.47	20.4 ± 2.0
(−)-pIV	412 ± 117	3.40 ± 0.51	28.1 ± 3.9
(−)-Vesamicol	13.0 ± 2.3	74.9 ± 18.6	421 ± 69
(+)-Vesamicol	289 ± 53	31.8 ± 11.4	359 ± 39
(+)-Pentazocine	—	19.9 ± 3.5	2680 ± 162
DTG	—	—	22.5 ± 4.2
Haloperidol	—	13.5 ± 2.0	110 ± 4

K_i values derived from IC_{50} values according to the equation, $K_i = IC_{50}/(1 + C/K_d)$, where C is the concentration of the radioligand and each K_d is the dissociation constant of the corresponding radioligand ((−)-[^{3}H]vesamicol to VAChT, [^{3}H]pentazocine to σ-1, [^{3}H]DTG to σ-2).
Values are means ± SEM of three experiments.
[a] K_d = 7.40 nM.
[b] K_d = 19.9 nM.
[c] K_d = 22.3 nM.

3. Results

Preparation of the (+)-pIV from 4-phenylpyridine via a six-step reaction produced overall yields of 21.2% (Fig. 1). Radiochemical yield, radiochemical purity and specific activity were estimated to be 40–75%, >95% and 600–1100 GBq/mmol, respectively.

Binding affinity of (+)-pIV and reference compounds to sigma receptors (σ-1, σ-2) and VAChT binding sites are shown in Table 1. pIV, as well as vesamicol, bound enantioselectively to the sigma receptors (σ-1, σ-2) and VAChT binding sites. The binding affinity of (+)-pIV to σ-1 and σ-2 was greater than that of (−)-pIV. Also, the binding affinity of (+)-pIV to VAChT binding sites was less than that of (−)-pIV. (+)-pIV (K_i = 1.30 nM) displayed more than 10 times greater affinity for the σ-1 receptor than (+)-pentazocine (K_i = 19.9 nM) or haloperidol (K_i = 13.5 nM), which are known as sigma ligands. (+)-pIV displayed a very low affinity for VAChT binding sites (K_i = 1260 nM).

The results of the saturation binding study of (+)-[^{125}I]pIV are shown in Figure 2 and Table 2. (+)-[^{125}I]pIV in the absence or presence of 1 μM pentazocine exhibited a K_d of 6.96 or 21.9 nM with a B_{max} of 799 fmol/mg of protein or 397 fmol/mg protein, respectively. The B_{max} of (+)-[^{125}I]pIV to the σ-1 receptor or σ-2 receptor is thought to be 402 fmol/mg protein or 397 fmol/mg protein, respectively.

The results of the inhibition of (+)-[^{125}I]pIV binding with various reference compounds are shown in Table 3. IC_{50} values for spiperone, ketanserin, noradrenaline and quinuclidinyl benzilate (QNB) exceeded 5 μM.

4. Discussion

Previously, we reported that radioiodinated (−)-*o*-iodovesamicol [(−)-oIV], radioiodinated at the *ortho*-position of the 4-phenylpiperidine moiety of (−)-vesamicol, was suitable as a VAChT mapping agent.[19] On the other hand, Efange et al.[20,22] and Custer et al.[21] reported that there were several vesamicol analogs showing great affinity for sigma receptors. In the process of developing a VAChT mapping agent, we found that

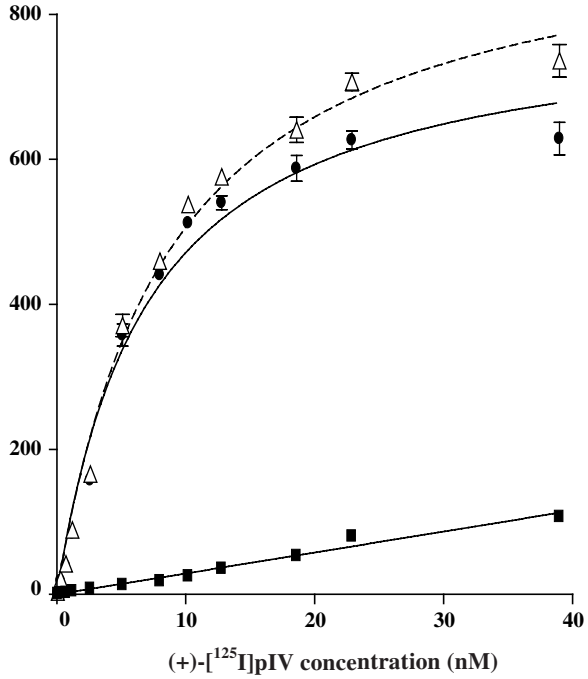

Figure 2. Equilibrium saturation binding of (+)-[^{125}I]pIV to rat cerebral membranes. (△) Total binding; (●) specific binding; (■) non-specific binding determined by the addition of 10 μM of (+)-pIV. K_d (6.96 ± 1.11 nM) and B_{max} (799 ± 44 fmol/mg of protein) values were calculated with the 'Graphpad Prism' computer program. Values are means ± SEM of three experiments.

Table 2. (+)-[^{125}I]pIV binding parameters (K_d, B_{max}) in rat brain membranes

	Pentazocine (1 μM)	
	In the absence	In the presence
K_d (nM)	6.96 ± 1.11	21.9 ± 2.1
B_{max} (fmol/mg protein)	799 ± 44	397 ± 20

Values are means ± SEM of three experiments.

Table 3. Inhibition of (+)-[^{125}I]pIV with various drugs in rat cerebral membranes

Drugs	IC_{50} (nM)
(+)-pIV	6.31 ± 1.19
DTG	71.1 ± 28.7
Pentazocine[a]	18.2 ± 6.0
Haloperidol	12.6 ± 5.3
Spiperone	$5.53 \times 10^3 \pm 1.21 \times 10^3$
Ketanserin	$1.64 \times 10^4 \pm 2.30 \times 10^3$
QNB	$9.78 \times 10^3 \pm 0.88 \times 10^3$
Noradrenaline	$>1.00 \times 10^5$

Values are means ± SEM of three experiments.
[a] The IC_{50} value exhibited a high affinity for pentazocine by the best fit of a two site competition. The IC_{50} value of low affinity for pentazocine was 2.40×10^3 nM.

both introduction of iodine into the 4-phenylpiperidine moiety of vesamicol and a kind of optical isomer had an influence on the binding affinity for VAChT and sigma receptors (σ-1, σ-2). Namely, there is a possibility that the introduction of iodine into the *para*-position into the 4-phenylpiperidine moiety of (+)-enantiomer of vesamicol causes its binding affinity for sigma receptors to increase, and its binding affinity for VAChT binding sites decrease. Therefore, we synthesized (+)-[^{125}I]pIV and evaluated the potential usefulness of (+)-[^{125}I]pIV as a radiotracer for sigma receptor studies. As we expected it, the dextrorotatory isomer (+)-pIV (K_i = 1.30 nM [σ-1], K_i = 20.4 [σ-2]) had a higher affinity for sigma receptors than not only the dextrorotatory isomer (+)-vesamicol (K_i = 31.8 nM [σ-1], K_i = 359 [σ-2]) but also the levorotatory isomer (−)-pIV (K_i = 3.40 nM [σ-1], K_i = 28.1 [σ-2]). On the other hand, (+)-pIV (K_i = 1260 nM) displayed a much lower affinity for VAChT than (−)-vesamicol (K_i = 13.0 nM) or (−)-pIV (K_i = 412 nM).

Furthermore, (+)-pIV displayed a more than 10 times greater affinity for the σ-1 receptor than superior σ-1 ligands such as (+)-pentazocine and haloperidol. Though (+)-pIV displayed a high affinity for the σ-2 receptor and the σ-2 receptor binding affinity of (+)-pIV (K_i = 20.4 nM) was comparable to that of DTG (K_i = 22.5 nM), the binding affinity of (+)-pIV to the σ-1 receptor (K_i = 1.30 nM) was about 16 times higher than the σ-2 receptor (K_i = 20.4 nM). The selectivity of (+)-pIV (approximately 16) for the σ-2 and σ-1 receptors (σ-2/σ-1) was lower than that of (+)-pentazocine (approximately 135). However, (+)-pentazocine is thought to bind to other neuroreceptors.[15] Furthermore, (+)-[^{125}I]pIV is also expected to pass through BBB and to accumulate in the rat brain significantly in vivo, because racemic (+/−)-[^{125}I]pIV was reported to show significant accumulation (about 3% of the injection dose) with prolonged retention in the rat brain previously.[24] On the other hand, (+)-[^{3}H]pentazocine showed the low accumulation (about 0.30% dose of the injection dose at 60 min post-injection) in the rat brain (date not shown). Judging from the above, (+)-[^{125}I]pIV may be used for comparison of σ-1 receptor studies in vitro with those in vivo.

Data derived from a saturation binding study using (+)-[^{125}I]pIV were analyzed using the 'Graphpad Prism' computer program. Though the graph provided from the date (Fig. 2) represents the best fit of a one-site model, it was apparent that the B_{max} of (+)-[^{125}I]pIV binding was the sum of the σ-1 and σ-2 receptors, under a high radioligand ((+)-[^{125}I]pIV) concentration (1–60 nM) condition. In fact, the B_{max} values (799 fmol/mg of protein) of (+)-[^{125}I]pIV binding to membranes of rat brain were higher than that of (+)-SKF10,047 binding to the σ-1 receptor in rat brain membranes[23]. Furthermore, the B_{max} of (+)-[^{125}I]pIV binding was lower in the presence (397 fmol/mg protein) of 1 μM pentazocine than in its absence (799 fmol/mg protein). Considering these results, it was thought that the value of 397 fmol/mg protein and 402 fmol/mg protein showed that B_{max} of (+)-[^{125}I]pIV to the σ-2 receptor and σ-1 receptor, respectively.

In competitive inhibition studies using (+)-[^{125}I]pIV (Table 3), (+)-[^{125}I]pIV exhibited a high affinity for σ-1 receptor and a much lower affinity for the dopamine, serotonin, noradrenaline, and acetylcholine receptors. These results showed that (+)-[^{125}I]pIV would be a radioligand for σ-1 receptor with a high affinity and selectivity.

5. Conclusion

Radioiodinated (+)-pIV exhibited a more than 10 times higher affinity to the σ-1 receptor than pentazocine and haloperidol and the selectivity of (+)-pIV for the σ-2 and σ-1 receptors (σ-2/σ-1) was high (approximately 16). In conclusion, radioiodinated (+)-pIV has superior potential as a radioligand for σ-1 receptor studies in vitro.

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