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Syntheses and in vitro evaluation of decalinvesamicol analogues as potential imaging probes for vesicular acetylcholine transporter (VAChT)

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ABSTRACT

A series of vesamicol analogues, *o*-iodo-*trans*-decalinvesamicol (OIDV) or *o*-bromo-*trans*-decalinvesamicol (OBDV), were synthesized and their affinities to vesicular acetylcholine transporter (VAChT) and σ receptors (σ-1, σ-2) were evaluated by in vitro binding assays using rat cerebral or liver membranes. OIDV and OBDV showed greater binding affinity to VAChT (K_i = 20.5 ± 5.6 and 13.8 ± 1.2 nM, respectively) than did vesamicol (K_i = 33.9 ± 18.1 nM) with low affinity to σ receptors. A saturation binding assay in rat cerebral membranes revealed that [^{125}I]OIDV had a single high affinity binding site with a K_d value of 1.73 nM and a B_{max} value of 164.4 fmol/mg protein. [^{125}I]OIDV revealed little competition with inhibitors, which possessed specific affinity to each σ (σ-1 and σ-2), serotonin (5-HT_{1A} and 5-HT_{2A}), noradrenaline, and muscarinic acetylcholine receptors. In addition, BBB penetration of [^{125}I]OIDV was verified in in vivo. The results of the binding studies indicated that OIDV and OBDV had great potential to be VAChT imaging probes with high affinity and selectivity.

1. Introduction

Cholinergic nerve disorder is one of the known pathophysiologies of Alzheimer's disease (AD).[1–3] In the dopamine neurons of early Parkinson's disease, it's known that the presynaptic changes represented by the dopamine transporter are bigger than the postsynaptic changes represented by the dopamine D_2 receptor.[4–6] In the cholinergic neurons of early AD, the presynaptic changes are also more remarkable than the postsynaptic changes. The decrease in the activity of choline acetyltransferase (ChAT),[7] which synthesizes acetylcholine (ACh), or vesicular acetylcholine transporter (VAChT),[8,9] which transports ACh into synaptic vesicles, are notable so that they are attractive targets of molecular imaging for the purpose of early detection of AD with single-photon emission computed tomography (SPECT) or positron emission tomography (PET).[10]

Vesamicol (2-(4-phenylpiperidin-1-yl)cyclohexanol), which is known as a selective antagonist for VAChT in cholinergic neurons, has attracted much attention as a potential VAChT imaging probe,[11–22] but the additional affinity for σ receptors (σ-1, σ-2)[23] is an obstacle to progress in this field. In 1989, Rogers et al. reported that *trans*-decalinvesamicol (DV) revealed a high affinity for VAChT among various vesamicol analogues in in vitro and in vivo binding assays (Fig. 1).[24] Previously, we developed (−)-*o*-iodovesamicol ((−)-oIV), which possessed high affinity for VAChT and selectivity against σ-1 or σ-2 receptors.[25–28] On the basis of these observations of DV and (−)-oIV, adequate vesamicol analogues for VAChT imaging were expected with a *trans*-decalin framework instead of the cyclohexane moiety and a halogen at the *ortho*-position of the 4-phenylpiperidine moiety. In this paper, we report the syntheses and evaluation of two newly designed vesamicol analogues, *o*-iodo-*trans*-decalinvesamicol (OIDV) and *o*-bromo-*trans*-decalinvesamicol (OBDV), in the development of a useful VAChT imaging probe with higher affinity and selectivity.

2. Results

2.1. Chemistry

4-(2-Bromophenyl)piperidine (**3**) was derived from *o*-bromobenzaldehyde (**1**) by four steps. (Scheme 1) coupling reaction of **3** with *trans*-decalin-2,3-oxide (**4**) furnished OBDV (**5**) in 57% yield. A bromo substituent of **5** was replaced by a trimethylstannyl group with $Pd(PPh_3)_4$ and hexamethylditin to obtain the key intermediate *o*-trimethylstannyl-*trans*-decalinvesamicol (OTDV) (**6**). Treatment of **6** with I_2 in $CHCl_3$ at room temperature succeeded in the introduction of an iodo substituent to give cold OIDV (**7**) in 80% yield. DV was prepared according to Roger's protocol.[24]

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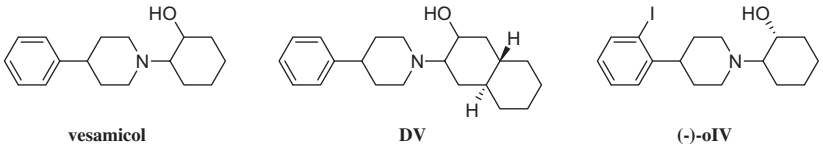
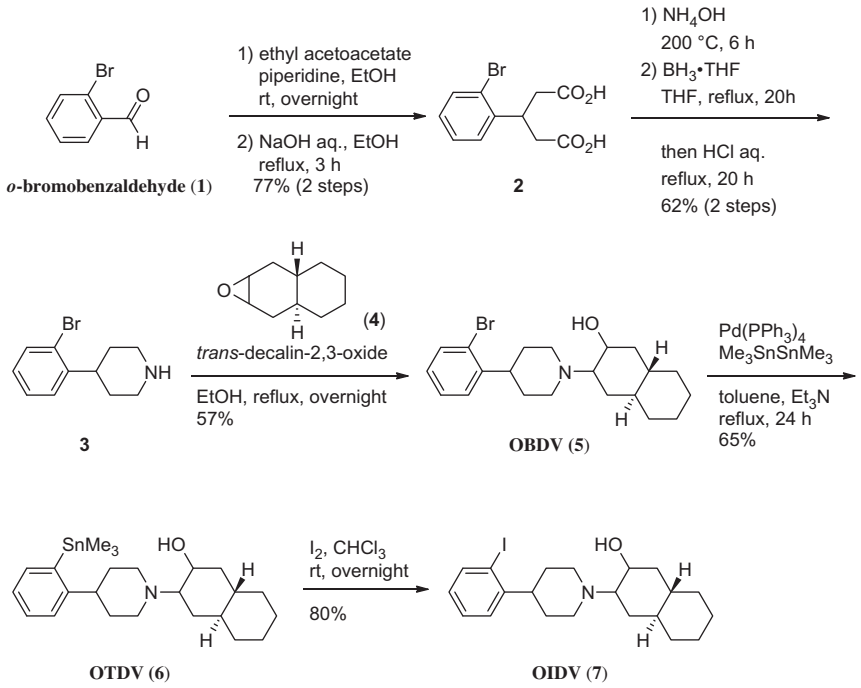


Figure 1. Vesamicol and its analogues.



Scheme 1. synthesis of OBDV, OTDV and OIDV.

2.2. In vitro binding assay

2.2.1. VAChT

The binding affinities (K_i values) of OBDV and OIDV to VAChT were measured by displacement of a typical VAChT antagonist (−)-[^{3}H]vesamicol (dissociation constant: K_d = 7.40 nM) in rat cerebral membranes using previously reported procedures.[26] To mask the σ receptors of the tissue, 1,3-di-*o*-tolylguanidine (DTG) was added into each assay tube. DV and vesamicol were also studied as comparable standards. The percent inhibition against the binding of (−)-[^{3}H]vesamicol to VAChT and the K_i values were calculated using GraphPad Prism v4 software. The inhibition constant K_i values were calculated from $IC_{50}/(1 + C/K_d)$, where C is the concentration of the radioligand. The results are summarized in Table 1. Each binding affinity to VAChT of DV, OBDV, and OIDV (K_i = 13.6 ± 8.8, 13.8 ± 1.2, and 20.5 ± 5.6 nM, respectively) was greater than that of vesamicol (K_i = 33.9 ± 18.1 nM).

Table 1
In vitro binding assay of vesamicol analogues

Entry	Compounds	K_i (nM)		
		VAChT	σ-1	σ-2
1	OIDV	20.5 ± 5.6	241.8 ± 98.9	118.8 ± 57.0
2	OBDV	13.8 ± 1.2	150.7 ± 62.9	137.5 ± 97.4
3	DV	13.6 ± 8.8	74.1 ± 39.9	68.3 ± 25.5
4	Vesamicol	33.9 ± 18.1	22.1 ± 3.6	86.7 ± 35.7
5	DTG	—	131.1 ± 39.2	31.7 ± 3.6
6	Pentazocine	—	12.1 ± 5.0	1880.8 ± 953.9
7	Haloperidol	—	3.5 ± 0.8	51.6 ± 14.6

2.2.2. σ-1 Receptor

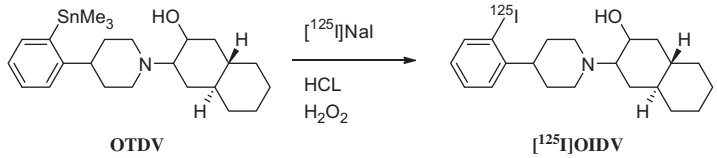
To evaluate selectivity against VAChT, OBDV and OIDV were screened for binding affinities to σ receptors using in vitro competitive binding assays. The binding affinities of OBDV and OIDV to the σ-1 receptor were measured by displacement of a σ-1 receptor selective agonist (+)-[^{3}H]pentazocine (K_d = 19.9 nM) in rat cerebral membranes using previously reported procedures.[26] DV, vesamicol, DTG, haloperidol, and non-radiolabeled pentazocine were also studied as comparable standards. The results are also summarized in Table 1. OBDV and OIDV (K_i = 150.7 ± 62.9 and 241.8 ± 98.9 nM, respectively) showed much lower affinity to the σ-1 receptor than did vesamicol (K_i = 22.1 ± 3.6 nM) or pentazocine (K_i = 12.1 ± 5.0 nM).

2.2.3. σ-2 Receptor

The binding affinities of OBDV and OIDV to the σ-2 receptor were measured by displacement of a σ receptor selective agonist [^{3}H]DTG (K_d = 22.3 nM) in rat liver membranes using previously reported procedures.[26] To mask the σ-1 receptor of the tissue, pentazocine was added to each assay tube. DV, vesamicol, pentazocine, haloperidol, and non-radiolabeled DTG were also studied as comparable standards. The results are also summarized in Table 1. OBDV and OIDV (K_i = 137.5 ± 97.4 and 118.8 ± 57.0 nM, respectively) showed lower affinity to the σ-2 receptor than did vesamicol (K_i = 86.7 ± 35.7 nM) or DTG (K_i = 31.7 ± 3.6 nM).

2.3. Radiolabeling

Radioiodination of [^{125}I]OIDV was achieved through the tin-iodine exchange reaction from the precursor OTDV (**6**) with



OTDV [125I]OIDV

Scheme 2. Radiosynthesis of [125I] OIDV.

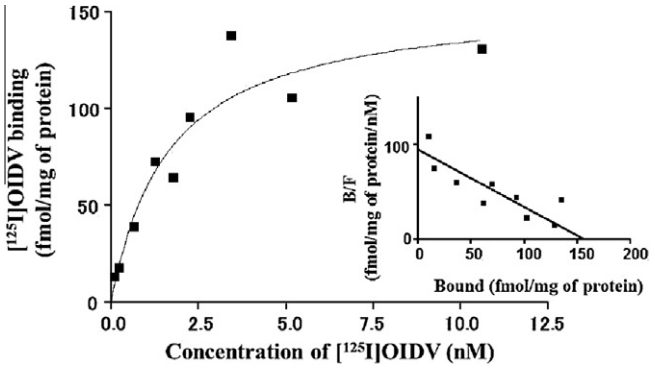


Figure 2. Saturation curve and scatchard plot of [125I] OIDV.

no-carrier-added [125I]NaI, HCl and H_2O_2. (Scheme 2) After purification by HPLC, the radiochemical yield and purity of [125I]OIDV were 80% (29.8 MBq) and 99%, respectively.

2.4. Partition coefficient determination

The partition coefficient ($\log P_{o/w}$) of [125I]OIDV was determined by the standard method with *n*-octanol and 0.1 M phosphate buffer. The partition coefficient was calculated with the followed formula: $\log P_{o/w} = \log_{10}$ (radioactivity in *n*-octanol layer/radioactivity in aqueous layer). The $\log P_{o/w}$ value of [125I]OIDV was 2.46 ± 0.02.

2.5. Saturation binding assay of [125I]OIDV

To investigate specific binding of [125I]OIDV to rat cerebral membranes, an in vitro saturation binding assay of [125I]OIDV was performed with haloperidol to mask the σ receptors. The resulting saturation curve and Scatchard plot are shown in Figure 2. The values of K_d and the maximum binding (Bmax) of [125I]OIDV, which were determined from the Scatchard plots with GraphPad Prism v4, were 1.73 nM and 164.4 fmol/mg of protein, respectively. We had already studied an in vitro saturation binding assay of (−)-[3H]vesamicol under almost the same conditions using DTG instead of haloperidol.[26] The K_d and B_{max} values of (−)-[3H]vesamicol were 7.40 nM and 163.7 fmol/mg of protein, respectively.

2.6. Inhibition of [125I]OIDV binding to rat cerebral membranes

Displacement of [125I]OIDV binding was performed by various ligands: pentazocine (a selective agonist for the σ-1 receptor), DTG (an agonist for the σ-1 and σ-2 receptors), spiperone (an antagonist for the serotonin 5-HT_{1A}, 5-HT_{2A}, and dopamine D_2 receptors), ketanserin (a selective antagonist for the serotonin 5-HT_{2A} receptor), noradrenalin (a ligand for the noradrenergic receptor), QNB (an antagonist for the muscarinic acetylcholine receptor), and vesamicol as a standard. The results are summarized in Table 2. The obtained K_i values (nM) of pentazocine (>10,000), DTG (1134), spiperone (1033), ketanserin (>10,000), noradrenalin (>10,000), and QNB (581.7) were much higher than that of vesamicol (32.9) except for OIDV (28.6) and OBDV (9.3).

Table 2
In vitro binding assay of [125I]OIDV

Entry	Compounds	K_i (nM)
1	Pentazocine	>10,000
2	DTG	1134
3	Spiperone	1033
4	Ketanserin	>10,000
5	Noradrenalin	>10,000
6	QNB	581.7
7	Vesamicol	32.9
8	OIDV	28.6
9	OBDV	9.3

2.7. Brain uptake of [125I]OIDV in in vivo studies

To evaluate the brain uptake of [125I]OIDV, the biodistribution studies were carried out using Sprague-Dawley rats (8 weeks, male, 250–300 g, $n = 4$). Data were calculated as percent of injected dose per gram of the tissue (% ID/g). At 10 min postinjection, the accumulation of [125I]OIDV in rat whole brain was 0.45 ± 0.04% ID/g.

3. Discussion

Since (−)-vesamicol is known to possess a high affinity for VAChT, its analogues are expected as potential VAChT probes. To develop them with high affinity and selectivity, we have studied various vesamicol analogues that have halogen at the *ortho*-, *meta*-, or *para*-position of the 4-phenylpiperidine moiety. We found that (−)-oIV and (−)-mIV have affinities for VAChT that were as great as (−)-vesamicol, although (+)-oIV, (+)-mIV, and (+)-pIV have low affinities for VAChT.[26] The absolute configuration of the vesamicol analogues must have a significant influence on the affinity for VAChT. However, vesamicol was reported to possess additional binding affinities for the σ receptors (σ-1, σ-2).[23] oIV and mIV also showed higher affinities for the σ-1 receptor than did (−)-vesamicol.[29] To acquire high VAChT selectivity against σ receptors, further modification of the molecular structure, especially part of cyclohexane, was required. Roger et al synthesized a number of vesamicol analogues, and examined their in vitro binding assay only for VAChT.[24] We guessed that DV, of which the affinity is higher than that of (−)-vesamicol in Roger's report, might have potential as the new VAChT imaging agent with great affinity and high selectivity. In fact, OIDV and OBDV, which have the *trans*-decalin moiety, showed higher affinity and selectivity for VAChT in in vitro than did vesamicol as standard. Since the affinities of oIV and mIV for VAChT were slightly lower than that of vesamicol in our previous report,[30] OIDV and OBDV were supposed to be more suitable for imaging probes of VAChT than oIV or mIV. OIDV or OBDV revealed good affinity and selectivity even as racemic compounds and we expected that the results would be improved after optical resolution. Unfortunately, however, optical resolution of them is more difficult than that of vesamicol. Experiments for optical resolution are now continuing.

OBDV displayed the greatest affinity for VAChT and higher selectivity against the σ-1 or σ-2 receptors as far as the results of in vitro binding assays, so it was expected to possess the potential for being a great VAChT probe for PET after radiolabeling with

^{76}Br. In addition, OIDV also has the potential to be a great VAChT probe for SPECT after radiolabeling with ^{123}I and, clinically, it is expected to be more useful because of its ease of use and handling. Consequently, OIDV radiolabeled with ^{125}I was chosen for further study of its characteristics. The typical tin-iodine exchange reaction afforded [^{125}I]OIDV in the sufficient radiochemical yield and purity (80% and 99%, respectively) without any noticeable byproducts. When formic acid was used instead of 0.1 N HCl in the radiolabeling reaction, the radiochemical yield was just around 30%. In the case of a stronger acid such as 1 N HCl, the radiochemical yield was 76%, but most of the remaining precursor OTDV turned into DV as a byproduct. The retention times of DV, [^{125}I]OIDV, and OTDV in the adopted HPLC condition were about 9, 12, and 18 min, respectively, so that [^{125}I]OIDV could be perfectly purified by HPLC.

An essential characteristic for in vivo imaging probes of the brain is to pass through the blood–brain barrier (BBB). Generally, lipophilicity is one of the important factors in the transport of chemical compounds through the BBB. Since [^{125}I]OIDV exhibited appropriate lipophilicity for BBB penetration ($\log P_{o/w}$ = 2.46 ± 0.02), it was expected to show adequate brain uptake after an intravenous injection.

Saturation binding assays of [^{125}I]OIDV in rat cerebral membranes were performed in order to investigate the binding property with haloperidol for masking the σ receptors (σ-1, σ-2). The specific binding was determined by subtracting the nonspecific binding from the total binding in rat cerebral membranes at each concentration of [^{125}I]OIDV (0.1–10 nM). The resulting saturation curve and the Scatchard plot are shown in Figure 2. A saturation binding assay in rat cerebral membranes indicated that [^{125}I]OIDV had a single high affinity binding site. [^{125}I]OIDV showed smaller K_d (1.73 nM) to VAChT than did (−)-[^{3}H]vesamicol (7.4 nM) or (−)-[^{125}I]oIV (17.4 nM).[26] This means that the binding of [^{125}I]OIDV is stronger than vesamicol or (−)-oIV. Since the B_{max} value of [^{125}I]OIDV (164.4 fmol/mg protein) is almost same as the B_{max} value of (−)-[^{3}H]vesamicol (163.7 fmol/mg protein), it is thought that the binding site of OIDV is the same as vesamicol except for σ receptors (σ-1, σ-2).

In the in vitro competitive binding assay of [^{125}I]OIDV as a radioligand, [^{125}I]OIDV did not compete with pentazocine, DTG, spiperone, ketanserin, noradrenalin, or QNB in rat cerebral membranes. In other words, it was verified that [^{125}I]OIDV possesses no affinity to the σ (σ-1 and σ-2), serotonin (5-HT_{1A} and 5-HT_{2A}), dopamine D_2, noradrenaline, or muscarinic acetylcholine receptors in rat cerebral membranes. In addition, the low K_i values of vesamicol or OBDV indicated that they occupied the binding sites of [^{125}I]OIDV. These results mean that the binding of [^{125}I]OIDV with rat cerebral membranes is specific for VAChT. DTG had just a weak influence on the binding of [^{125}I]OIDV in rat cerebral membranes, although OIDV revealed middle affinity for σ-2 receptors in in vitro binding assay using [^{3}H]DTG and rat liver membranes. We guess that it is because of the binding superiority of [^{125}I]OIDV–VAChT in comparison with σ-2 receptor and the difference of tissues, rat brain or liver. From these results, [^{125}I]OIDV is expected to bind to VAChT in brain preferentially, although σ-1 and σ-2 receptors present in rat brain widely.

In in vivo studies of [^{125}I]OIDV, the accumulation of [^{125}I]OIDV was detected in rat whole brain (0.45 ± 0.04% ID/g) at 10 min postinjection. We expected that [^{125}I]OIDV passed through the BBB. Further detailed in vivo evaluations of [^{125}I]OIDV are now continuing.

4. Conclusion

We have developed new VAChT imaging probes based on vesamicol, which had the framework of DV and the halogen at the *ortho*-position of the 4-phenylpiperidine moiety. These vesamicol analogues, OIDV and OBDV, showed high affinity and selectivity for VAChT in in vitro binding assay. [^{125}I]OIDV was derived from OTDV in high radiochemical yield and purity and revealed no affinity for various receptors in rat cerebrum except for VAChT. In addition, BBB penetration of [^{125}I]OIDV was verified in in vivo. From these results, [^{125}I]OIDV is thought to be a superior radioligand for VAChT in in vitro binding assay instead of (−)-[^{3}H]vesamicol. OIDV and OBDV are also expected as potential superb VAChT imaging agents after radiolabeling with ^{123}I or ^{76}Br. Further studies are now underway to evaluate their in vivo characteristics, examine after optical resolution, and optimize their structure for PET imaging.

5. Experimental

5.1. Syntheses

5.1.1. 4-(2-Bromophenyl)piperidine (3)

A solution of *o*-bromobenzaldhyde (20.0 g, 108 mmol), ethyl acetoacetate (27.3 mL, 216 mmol), and piperidine (2.14 mL, 21.6 mmol) in EtOH (77.2 mL) was stirred for 15 h at room temperature. To the reaction solution was added 12 M NaOH (360 mL) and EtOH (350 mL). The reaction mixture was refluxed for 4 h, cooled to room temperature, and the organic solvents removed. The residue was acidified with concd HCl (450 mL) in an ice bath, extracted with AcOEt, washed with water and brine, and concentrated to dryness. The residue was transferred to a glass filter and washed with cold Et_2O to obtain dicarboxylic acid **2** (23.9 g) as a white solid without further purification.

The above **2** (23.9 g, 83.2 mmol) was dissolved in 28% ammonia solution (280 mL) by heating, and then water was evaporated off under reduced pressure. The resulting residue was heated to 200 °C to melt for 6 h, cooled to room temperature, extracted with CH_2Cl_2, washed with Na_2CO_3 aq and brine, concentrated to dryness, and washed with cold Et_2O. BH_3·THF (1.0 M THF solution; 208 mL, 208 mmol) was added dropwise to a solution of the crude cyclic imide in THF (166 mL) at 0 °C under Ar. The reaction mixture was allowed to gradually reach room temperature and refluxed for 20 h. concd HCl aq (300 mL) was carefully added to the reaction mixture at 0 °C. The reaction mixture was refluxed for an additional 20 h, neutralized with NaOH aq, extracted with AcOEt, washed with water and brine, and concentrated to dryness. Solvents were evaporated off. Et_2O (1 L) was added to the residue at 0 °C. The resulting white precipitate was isolated by filtration and washed with cold Et_2O to obtain **3** (12.4 g, 48% from 1) as a white solid without further purification: ^{1}H NMR δ 7.54–7.51 (m, 1H), 7.28–7.26 (m, 2H), 7.07–7.00 (m, 1H), 3.21–3.17 (m, 2H), 3.10 (tt, 1H, J = 3.7, 11.9 Hz), 3.10 (dt, 2H, J = 2.3, 11.9 Hz), 2.21–2.19 (m, 1H), 1.87–1.83 (m, 2H), 1.65–1.53 (m, 2H); EI MS m/z 240 (M^++1, 85.3), 241 (M^+, 73.6).

5.1.2. *o*-Bromo-*trans*-decalinvesamicol (OBDV) (5)

A mixture of **3** (2.25 g, 9.36 mmol) and *trans*-decalin-2,3-oxide (2.14 g, 14.0 mmol) in EtOH (9.4 mL) was refluxed for 20 h. The solvent was evaporated off. The residue was chromatographed on silica gel with hexane-AcOEt (5:1) to give **5** (2.10 g, 57%) as a white solid: ^{1}H NMR δ 7.54–7.52 (m, 1H), 7.30–7.25 (m, 2H), 7.06–7.02 (m, 1H), 4.10–4.07 (m, 1H), 3.14–3.11 (m, 2H), 3.10 (tt, 1H, J = 3.4, 12.0 Hz), 2.33–2.30 (m, 1H), 2.21–2.16 (m, 1H), 2.12–2.07 (m, 1H), 1.92–1.19 (m, 17H), 1.03–0.91 (m, 2H); ^{13}C NMR δ 144.9, 132.8, 127.6, 127.5, 127.3, 124.5, 67.0, 64.7, 52.9, 49.4, 41.8, 36.7, 36.5, 35.9, 33.8, 33.7, 32.6, 32.5, 29.4, 26.6, 26.5; DART MS m/z 392 (M^++1, 50.8), 394 (M^++1, 47.3).

5.1.3. *o*-Trimethylstannyl-*trans*-decalinvesamicol (OTDV) (6)

A mixture of **5** (42.1 mg, 0.107 mmol), $Pd(PPh_3)_4$ (7.5 mg, 6.44×10^{-3} mmol) and hexamethylditin (87.9 mg, 0.268 mmol) in anhydrous toluene (1.1 mL) was refluxed for 15 h under Ar. The solvent was evaporated off. The residue was chromatographed on silica gel with hexane-AcOEt (10:1) to give **6** (33.4 mg, 65%) as a white solid: ^{1}H NMR δ 7.18–7.15 (m, 1H), 7.09–7.04 (m, 2H), 6.95–6.90 (m, 1H), 3.86–3.81 (m, 1H), 2.94–2.86 (m, 2H), 2.15–0.65 (m, 2^3H), 0.06 (m, 9H); ^{13}C NMR δ 153.02, 141.65, 135.95, 128.80, 125.79, 125.65, 66.94, 64.65, 53.11, 49.85, 47.21, 36.62, 36.49, 35.85, 34.57, 34.54, 33.75, 33.62, 29.46, 26.55, 26.48, −8.22; DART MS *m*/*z* 478 (M^++1, 100).

5.1.4. *o*-Iodo-*trans*-decalinvesamicol (OIDV) (7)

Iodine (405 mg, 1.60 mmol) was added to a reaction mixture of **6** (253 mg, 0.531 mmol) in $CHCl_3$ (1.1 mL). The reaction mixture was stirred for 24 h at the same temperature, quenched by addition of saturated aqueous $Na_2S_2O_3$ and saturated aqueous $NaHCO_3$, extracted with $CHCl_3$, washed with water and brine, dried, and concentrated to dryness. The residue was chromatographed on silica gel with hexane-AcOEt (10:1) to give **7** (187 mg, 80%) as a white solid: ^{1}H NMR δ 7.84–7.82 (m, 1H), 7.33–7.23 (m, 2H), 6.91–6.87 (m, 1H), 4.10–4.07 (m, 1H), 3.14–3.11 (m, 2H), 2.79 (tt, 1H, *J* = 3.4, 12.0 Hz), 2.33–2.30 (m, 1H), 2.22–2.16 (m, 1H), 2.12–2.07 (m, 1H), 1.89–1.19 (m, 17H), 1.03–0.94 (m, 2H); ^{13}C NMR δ 151.8, 147.9, 139.6, 128.5, 127.9, 126.7, 67.02, 64.6, 52.8, 49.4, 47.0, 36.7, 36.5, 36.0, 33.9, 33.7, 32.9, 32.8, 29.4, 26.6, 26.5; DART MS *m*/*z* 440 (M^++1, 84.4).

5.2. Preparation of rat cerebral and liver membranes

Animal experiments were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi Campus of Kanazawa University. Sprague-Dawley rat (8 weeks, male, 250–300 g) cerebrum or livers were homogenized in ice-cold 0.32 M sucrose with a Teflon-glass homogenizer. The homogenate was centrifuged at 1000*g* at 4 °C for 10 min. The resulting precipitate was removed and the supernatant was centrifuged at 55000*g* at 4 °C for 1 h. Cerebral membranes needed additional steps in that the resulting precipitate was suspended in ice-cold water with a homogenizer and again centrifuged at 55000*g* at 4 °C for 1 h. Each resulting pellet of rat cerebrum or liver was suspended in an ice-cold buffer (50 mM Tris–HCl/0.32 M sucrose, pH 7.8) with a homogenizer.

5.3. In vitro binding assay

5.3.1. VAChT

(−)-[^{3}H]Vesamicol (K_d = 7.40 nM) was used as a radioligand for the VAChT receptor. Rat cerebral membranes was added to each ice-cold assay tube containing (−)-[^{3}H]vesamicol, DTG (0.2 μM) to mask the σ receptors and the displacing ligands at various concentrations (1.0×10^{-10}–10^{-5} M) in 50 mM Tris–HCl buffer (pH 7.8) in quadruplicate. After the addition of the tissue in an ice bath, each reaction mixture in tubes was incubated at 37 °C for 60 min, and then quenched by cooling in an ice bath. Each sample was passed through glass-microfiber filters (Whatman, GF/F), which were presoaked in 0.3% polyethyleneimine, using a cell harvester. The filters were washed with 50 mM Tris–HCl buffer (pH 7.8) and their radioactivities were counted with a liquid scintillation counter (Aloka, LSC-5100). Three independent experiments were carried out.

5.3.2. σ-1 Receptor

(+)-[^{3}H]Pentazocine (K_d = 19.9 nM) was used as a specific radioligand for the σ-1 receptor. Rat cerebral membranes was added to each ice-cold assay tube containing the above radioligand and the displacing ligands at various concentrations (1.0×10^{-10}–10^{-5} M) in 50 mM Tris–HCl buffer (pH 7.8) in quadruplicate. After the addition of the tissue in an ice bath, each reaction mixture in tubes was incubated at 37 °C for 90 min, and then quenched by cooling in an ice bath. Each sample was passed through glass-microfiber filters (Whatman, GF/B), which were presoaked in 0.3% polyethyleneimine, using a cell harvester. The filters were washed with 50 mM Tris–HCl buffer (pH 7.8), and their radioactivities were counted with a liquid scintillation counter (Aloka, LSC-5100). Three independent experiments were carried out.

5.3.3. σ-2 Receptor

[^{3}H]DTG (K_d = 22.3 nM) was used as a radioligand for the σ-2 receptor with pentazocine to mask the σ-1 receptor. Rat liver membranes was added to each ice-cold assay tube containing [^{3}H]DTG, pentazocine (1.0 μM), and the displacing ligands at various concentrations (1.0×10^{-10}–10^{-5} M) in 50 mM Tris–HCl buffer (pH 7.8) in quadruplicate. After the addition of the tissue in an ice bath, each reaction mixture in tubes was incubated at 37 °C for 90 min, and then quenched by cooling in an ice bath. Each sample was passed through glass-microfiber filters (Whatman, GF/B), which were presoaked in 0.3% polyethyleneimine, using a cell harvester. The filters were washed with 50 mM Tris–HCl buffer (pH 7.8), and their radioactivities were counted with a liquid scintillation counter (Aloka, LSC-5100). Three independent experiments were carried out.

5.4. Radiolabeling

To a solution of HCl (0.1 N, 50 μL) and **6** (1 mg/mL, 50 μL) in a vial, [^{125}I]NaI (carrier-free, 25 μL, 37.1 MBq) and 3% H_2O_2 (50 μL) were added. The reaction mixture was shaken at room temperature for 1 min, left for 20 min, quenched by addition of $Na_2S_2O_5$ (200 mg/mL; 50 μL), turned basic by addition of NaOH (1 N, 50 μL), diluted with H_2O (5 mL), filtered through a Sep-Pak C18 (Waters, Light), which was pre-washed with EtOH (5 mL) and H_2O (15 mL). The Sep-Pak C18 containing [^{125}I]OIDV was washed with H_2O (5 mL) to remove unreacted [^{125}I]NaI or any salts. [^{125}I]OIDV in the Sep-Pak C18 was eluted with EtOH (5 mL) and concentrated to a 100-μL solution. The solution was then purified by a reverse phase HPLC column (Zorbax-ODS RX-C18, 9.6 × 250 mm) warmed to 40 °C. The retention time of [^{125}I]OIDV was about 12 min with the mobile phase consisting of 90:10:0.2 v/v/v acetonitrile/H_2O/monoethanolamine at a 4.0 mL/min flow rate. The radiolabeling was repeated six times.

5.5. Partition coefficient determination

[^{125}I]OIDV (0.51 MBq) was added to a mixture of *n*-octanol (15 mL) and 0.1 M phosphate buffer (pH 7.4; 15 mL) in a test tube (*n* = 4). The test tube was vortexed for 5 min at room temperature, followed by centrifugation for 10 min (2000*g*). The radioactivities of four samples (1.0 mL) from each layer were measured with an auto well gamma counter (Aloka, AccuFLEX γ7010). The independent experiments were repeated three times.

5.6. Saturation binding assay of [^{125}I]OIDV

Saturation analysis was performed by incubation in various concentrations of [^{125}I]OIDV (0.1–10 nM) with rat cerebral membranes (400 μg protein) and 100 nM haloperidol using 50 mM Tris–HCl buffer (pH 7.8) for 1 h at 37 °C in quadruplicate. Each sample was passed through glass-microfiber filters (Whatman,

GF/F), which were presoaked in 0.3% polyethyleneimine, using a cell harvester. The filters were washed with 50 mM Tris–HCl buffer (pH 7.8) and their radioactivities were counted with an auto well gamma counter (Aloka, AccuFLEX γ7010). Nonspecific binding was determined with non-radiolabeled OIDV (10 μM). Three independent experiments were carried out.

5.7. In vitro binding assay of [^{125}I]OIDV

Rat cerebral membranes membranes was added to each ice-cold assay tube containing 5 nM [^{125}I]OIDV and each ligand (pentazocine, DTG, spiperone, ketanserin, noradrenalin, QNB, vesamicol, OIDV, or OBDV) at various concentrations (10^{-10}–10^{-5} M) in 50 mM Tris–HCl buffer (pH 7.8) in quadruplicate. Nonspecific binding was determined at a concentration of 10 μM OIDV. After the addition of the tissue, the tubes were removed from the ice, and incubated at 37 °C for 1 h, quenched by cooling in an ice bath. Each sample was passed through glass-microfiber filters (Whatman, GF/F), which were presoaked in 0.3% polyethyleneimine, using a cell harvester. The filters were washed with 50 mM Tris–HCl buffer (pH 7.8), and their radioactivities were counted with an auto well gamma counter (Aloka, AccuFLEX γ7010).

5.8. Brain uptake of [^{125}I]OIDV in in vivo studies

The [^{125}I]OIDV (0.4 mL, 185 kBq) was injected intravenously via the tail vein into Sprague-Dawley rats (8 weeks, male, 250–300 g, $n = 4$). At 10 min postinjection, the rats were sacrificed. Their whole brains were taken out and weighed. The radioactivity of each sample was counted with an auto well gamma counter (Aloka, AccuFLEX γ7010). Data were calculated as percent of injected dose per gram of the tissue (% ID/g).

Supplementary data

Supplementary data (HPLC charts of OBDV and OIDV) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.06.040. These data include MOL files and InChiKeys of the most important compounds described in this article.

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