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Vesamicol and some of its derivatives: Questionable ligands for selectively labelling acetylcholine transporters in rat brain

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Abstract

Presynaptic cholinergic markers could be used for estimating the integrity of the cholinergic systems in the human brain with brain imaging techniques such as Single-photon emission computed tomography (SPECT). Vesamicol, an inhibitor of the vesicular acetylcholine transporter, and some of its derivatives have been suggested as potential ligands for this purpose. However, vesamicol binds not only to acetylcholine transporters but also to σ binding sites. In the present study, we estimated the contribution of σ site labelling to [^{3}H](−)-vesamicol binding in different rat brain regions by selectively labelling the acetylcholine transporter, using [^{3}H](−)-vesamicol in the presence of the σ-ligand 1,3-di(2-tolyl)guanidine to occlude the σ binding sites. The contribution of σ site labelling was substantial in all brain regions and ranged from 25% in the striatum to 60% in the medulla. In addition, we investigated, in various experimental set ups, the affinities of several vesamicol derivatives for acetylcholine transporters and σ binding sites. All vesamicol derivatives used displayed a higher affinity for the σ_1 site than for the acetylcholine transporter and also displayed a high σ_2 site affinity. This poor selectivity limits the usefulness of these compounds as selective cholinergic markers for brain imaging studies. © 1997 Elsevier Science B.V.

Keywords: Vesamicol; AH5183; Alzheimer's disease; Acetylcholine transporter; σ binding site; SPECT (Single-photon emission computed tomography)

1. Introduction

Alzheimer's disease is the most common form of dementia, with an incidence that is highly age dependent. The extensive loss of cholinergic neurones found in the autopsied brains of Alzheimer's disease patients led to the cholinergic hypothesis, which postulates a connection between some of the cognitive impairments of this disorder — especially the loss of memory — and a disturbance in cholinergic neurotransmission (Perry, 1986). Because the loss of cholinergic neurones seems to correlate both with neuropathological variables, such as plaque density, and with the extent of the cognitive deficits, the use of drugs that increase cholinergic activity has been encouraged (Davis and Powchik, 1995). Clinical findings with acetylcholinesterase inhibitors (for instance tacrine and physostigmine) have indicated that this class of compounds produces a modest improvement of cognitive and especially memory functions (Mohs et al., 1985; Davis et al., 1992). However, the results of studies with acetylcholinesterase inhibitors also indicate the existence of various subgroups of patients, possibly with different degrees of cholinergic deficiency, since some patients responded much more adequately than others (Davis and Powchik, 1995).

The development of a radioligand as a selective marker of cholinergic neurones for Single-photon emission computed tomography (SPECT) would enable the visualization of the nerve terminal density of cholinergic neurones in the brain in vivo. This approach might provide insight not only into the possible existence of subpopulations of Alzheimer's disease patients in terms of differences in cholinergic deficiency, but also into the progression of the loss of cholinergic neurones in the brains of patients on a longitudinal basis.

In cholinergic neurones, newly synthesized acetylcholine is transported from the cytoplasm into synaptic vesicles for storage and subsequent impulse-driven quantal release. Vesamicol, or AH5183 (2-(4-phenylpiperidino)cyclohexanol, **1**, see Fig. 1) is known as

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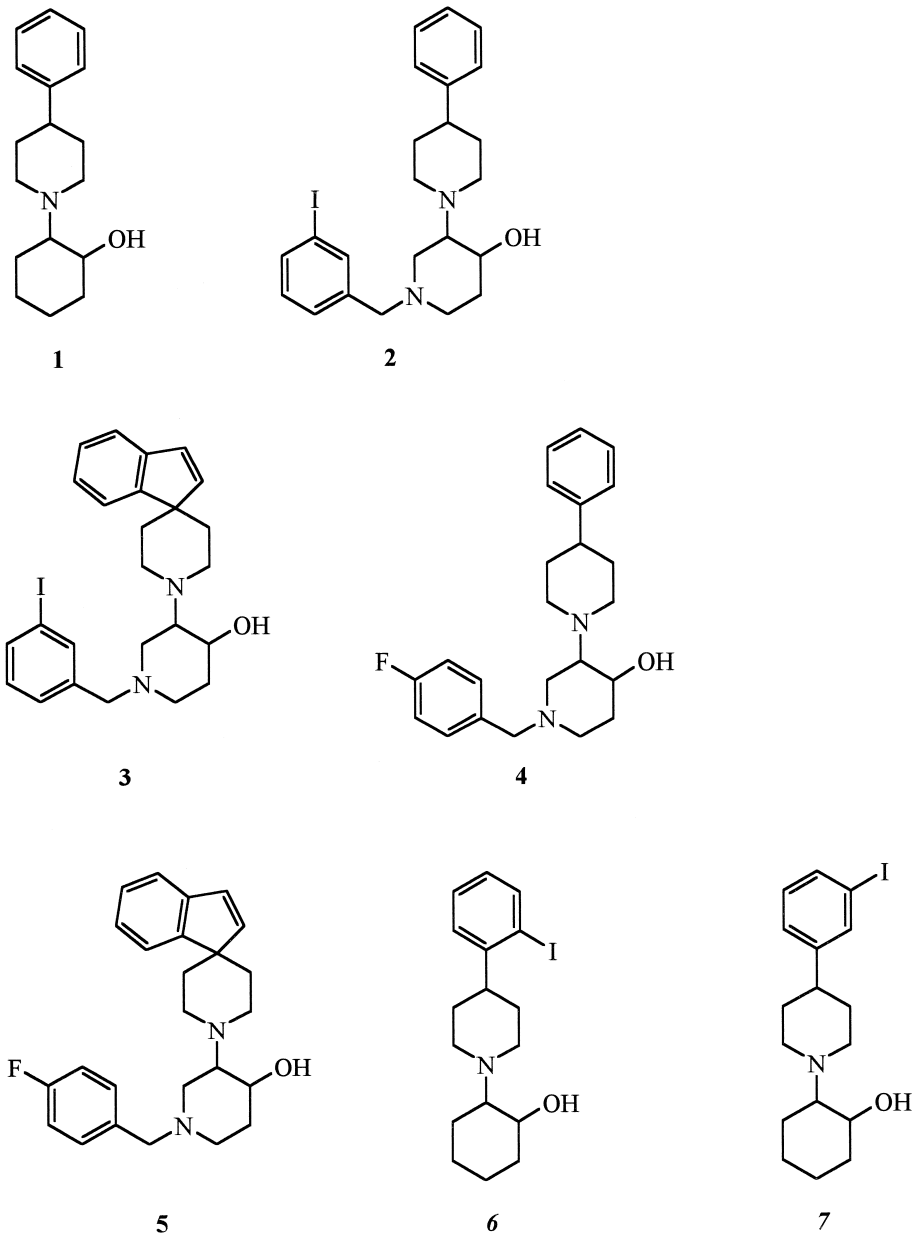

Fig. 1. Structures of vesamicol (**1**) and its derivatives.

an inhibitor of this vesicular acetylcholine transport (Marshall and Parsons, 1987). It binds with high affinity to the vesicular acetylcholine transporter, which makes it in principle an appropriate ligand for mapping of the central cholinergic pathways in the mammalian brain. However, lesioning of the septum, a brain nucleus where cholinergic cell bodies are localized, resulted in a smaller loss of [^{3}H](−)-vesamicol binding sites in the neocortex and hippocampus (both projection areas of the septum) compared with the loss of choline acetyltransferase activity, an enzyme that is specifically localized in cholinergic synapses (Altar and Marien, 1988). Similar discrepancies were found in the amygdala of Alzheimer's disease patients (Kish et al., 1990) and in the temporal cortex of patients with Alzheimer's disease and Parkinson's disease (Ruberg et al., 1990). A possible reason for these discrepancies is that [^{3}H](−)-vesamicol also binds to σ binding sites (Efange et al., 1995). These σ binding sites are likely to be present on various cells other than cholinergic neurones.

The primary aim of the present study was to investigate the specificity of vesamicol binding to the vesicular acetylcholine transporter in membrane preparations of various rat brain regions. We developed a radioligand binding assay to selectively label the vesicular acetylcholine transporter, using [^{3}H](−)-vesamicol in the presence of 1,3-di(2-tolyl)guanidine (DTG) to occlude the labelling of σ binding sites, and calculated K_d and B_{max} values. We also investigated the specificity of vesamicol derivatives (and

especially iodine-containing vesamicol derivatives as potential SPECT ligands) for the cholinergic transporters as compared to σ sites. σ_2 Site binding was determined by measuring [^{3}H]DTG binding to cultured fusion cells (N18TG2 neuroblastoma cells fused with primary septal neuron cells of the rat, Lee et al., 1990) and σ_1-site binding was estimated in guinea pig brain tissue (medulla oblongata) with [^{3}H]haloperidol (DeLoore et al., 1994).

2. Materials and methods

2.1. Compounds

Vesamicol derivatives were kindly donated by Dr. S.M.N. Efange (Department of Radiology, University of Minnesota, Minneapolis, MN, USA) and Dr. K. Shiba (Radioisotope Center Kanazawa University, Kanazawa, Japan); (+)-3(3-hydroxyphenyl)-*N*-(1-propyl)piperidine {(+)-3-PPP} was purchased from Astra (Södertalje, Sweden); (+)- and (−)-vesamicol were purchased from Research Biochemicals International (Natick, MA, USA). DTG was purchased from Aldrich (Milwaukee, WI, USA); [^{3}H]haloperidol (15 Ci/mmol, 0.55 TBq/mmol) was obtained from the Janssen Research Foundation (Beerse, Belgium). [^{3}H]DTG (35 Ci/mmol, 1.3 TBq/mmol) and [^{3}H](−)-vesamicol (31 Ci/mmol, 1.2 TBq/mmol) were purchased from New England Nuclear (Brussels, Belgium). Test compounds were dissolved and diluted in pure dimethylsulfoxide (DMSO). Just prior to addition to the incubation mixture a final dilution of 1/20 in assay buffer was made.

2.2. Membrane preparation

Rats (female Wistar 180–200 g) or guinea pigs (male Dunkin–Hartley, 300 g) were decapitated, the brains were rapidly removed and various brain regions were dissected (Paxinos and Watson, 1986). The tissue was homogenized in buffer at 4°C, using a Duall homogenizer (conical glass tube and Teflon pestle), in 25 volumes per wet weight of tissue (v/w) of 50 mM Tris–HCl, pH 7.4. The crude homogenate was centrifuged for 10 min at 16 000 × *g* in a refrigerated Sorval RC-5B centrifuge and the pellet was resuspended in ice-cold 50 mM Tris–HCl, pH 7.4, by vortexing. Membrane pellets were washed twice by resuspension and centrifugation to remove endogenous inhibitors. The final pellet was suspended in 10 v/w of ice-cold 50 mM Tris–HCl, pH 7.4, by vortexing. This membrane suspension was further diluted 8 and 10 × with assay buffer for guinea pig and rat brain preparations, respectively.

2.3. Radioligand binding assays

The incubation mixture contained 400 μl membrane suspension, 50 μl of the radioligand and 50 μl assay buffer (for total binding) or 50 μl of the test compound (for specific binding). For inhibition experiments non-labelled compounds were added at 8 to 10 concentrations in a range spanning two orders of magnitude lower and higher than the IC_{50} value (concentration producing 50% inhibition of the specific binding of the radioligand). Mixtures were incubated and reactions were stopped by the addition of 5 ml of ice-cold assay buffer, followed by rapid filtration under vacuum through Whatman GF/B glass fibre filters (presoaked in 0.1% polyethylenimine). Filters were washed twice with 5 ml of ice-cold assay buffer and the radioactivity retained on the filter was measured in counting vials with 2 ml of scintillation fluid (Packard ultima gold MV) in a Packard Tricarb liquid scintillation spectrometer with a counting efficiency of 60%. Counts are expressed in disintegrations per minute (dpm) with reference to experimental quench curves.

2.4. [^{3}H](−)-vesamicol binding assay

The assay buffer contained 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4. Rat brain membranes were incubated with [^{3}H](−)-vesamicol in 0.5 ml assay buffer for 30 min at 37°C in the absence (total binding) or the presence (non specific binding) of 10 μM (−)-vesamicol. For ligand concentration binding isotherms a range of concentrations between 2 and 100 nM [^{3}H](−)-vesamicol was used and for competition binding experiments a concentration of 4 nM [^{3}H](−)-vesamicol was used. For occlusion of σ-sites 200 nM DTG was added.

2.5. σ_1 receptor binding

Experiments were performed using guinea pig medulla oblongata membranes. Brain membranes were incubated with 1 nM [^{3}H]haloperidol in 0.5 ml of 50 mM Tris–HCl (pH 7.7) for 60 min at 25°C in the absence (total binding) or the presence (nonspecific binding) of 10 μM (+)-3-PPP.

2.6. σ_2 binding assay

Fusion cells (N18TG2 neuroblastoma cells fused with primary septal neuron cells of the rat, Lee et al., 1990) were cultured in petri dishes for 5 days. These cells were washed twice with phosphate-buffered saline, scraped off, collected and centrifuged for 10 min at 3000 × *g*. The pellet was placed in ice-cold hypotonic buffer (10 mM Tris–HCl, pH 7.7), homogenized for 10 s with a Duall homogenizer and centrifuged for 30 min at 27 000 × *g* in a refrigerated Sorval RC-5B centrifuge. The pellet was washed once with 50 mM Tris–HCl buffer, pH 7.7, by suspension followed by centrifugation, and was finally resuspended at a concentration of 0.15–0.25 mg protein/ml in the same buffer. This membrane suspension was incubated with 4 nM [^{3}H]DTG in 0.5 ml of 50 mM Tris–HCl

(pH 7.7) for 90 min at 25°C in the absence (total binding) or the presence (nonspecific binding) of 10 μM haloperidol.

2.7. Data analysis

For the inhibition experiments sigmoidal inhibition curves were fitted by nonlinear regression analysis, using the computer program Graphpad Prism (Graphpad Software, San Diego, CA, USA). IC_{50} values were derived from the curve; pIC_{50} values ± S.D. were calculated. K_i values were calculated according to $K_i = IC_{50}/(1 + C/K_d)$ with C is the concentration and K_d the equilibrium binding constant of the radioligand. For ligand concentration binding curves, curve fitting to a rectangular hyperbola was performed by nonlinear regression analysis (Graphpad Prism). K_d and B_{max} values (B_{max}: maximal of binding sites in the membrane preparation) were derived from the calculated curve. All experiments were performed and analyzed three times independently.

3. Results

Because vesamicol also shows high affinity for σ binding sites, we selected DTG, a ligand for both the σ_1 and σ_2 binding sites (Weber et al., 1986), to test its concentration-dependent displacement of vesamicol binding. Fig. 2 shows the concentration dependency of [^{3}H](−)-vesamicol displacement by DTG in rat brain striatum labelled with 4 nM [^{3}H](−)-vesamicol. The DTG inhibition curve showed a highly significant correlation ($R^2 = 0.96$) to a two-site fit ($F(2,13) = 3.92$, $P < 0.05$), which indicates that [^{3}H](−)-vesamicol also has affinity for σ binding sites and that these binding sites are present in the rat striatum. The K_i values (average of five independent experiments) for DTG for the two binding sites were 21 ± 2 nM and 1.1 ± 0.1 μM, respectively. Based on this experiment and on separately measured binding affinities of DTG for σ binding sites (K_i value was 18.4 nM for the σ_1 and 16.4 nM for the σ_2 binding site (see Table 2), we concluded that the high- and low-affinity binding sites for DTG reflect σ binding sites (approx. 31%) and the acetylcholine transporter (approx. 69%), respectively. Therefore, we added 200 nM DTG to the vesicular acetylcholine transporter binding assay in order to occlude the σ binding sites. The vesicular acetylcholine transporter binding sites seemed to be hardly occupied at this concentration.

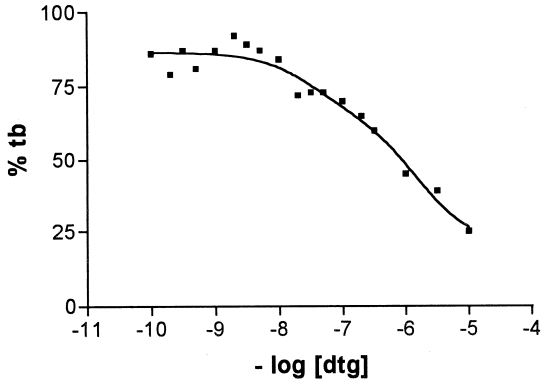

Fig. 2. Inhibition of [^{3}H](−)-vesamicol (4 nM) binding to rat striatum membranes by DTG. Fitting to a two-site curve yielded a high-affinity site $pIC_{50} = 7.58$ (31% of specific binding) and a low-affinity site $pIC_{50} = 5.90$ (69% of specific binding). The competition binding curve was significantly better described by a two-site model ($F(2, 13) = 3.92$, $P < 0.05$). Goodness of fit: correlation coefficient $R^2 = 0.96$. Data displayed were obtained from five independent experiments.

The K_d and B_{max} values derived from the [^{3}H](−)-vesamicol concentration binding curves for various regions of the rat brain, in the presence and the absence of DTG, are shown in Table 1. From these results it is obvious that there was marked σ site binding in the absence of DTG. The contribution of σ site labelling was very high (> 50%) in the cortex, medulla oblongata and cerebellum. A substantial number of σ binding sites were also observed in the hippocampus (47%) and striatum (25%). An example of a [^{3}H](−)-vesamicol concentration binding curve for rat cortex in the presence and the absence of DTG is shown in Fig. 3. Since (−)-vesamicol shows affinities in the same order of magnitude for the vesicular acetylcholine transporter and for both σ sites (see Table 2), adding 200 nM DTG does not change the affinity of binding for [^{3}H](−)-vesamicol but reduces only the number of available binding sites.

To thoroughly evaluate the properties of different

Table 1
Parameters of specific [^{3}H](−)-vesamicol binding in the absence of DTG and in the presence of 200 nM DTG in rat brain regions (mean values ± S.D., $n = 3$)

	No DTG		200 nM DTG		percent occluded sites by DTG (%)
	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)	
Striatum	23.9 ± 4.2	1927 ± 319	29.9 ± 11.4	1444 ± 315	25
Hippocampus	22.8 ± 8.0	1289 ± 280	22.7 ± 4.5	687 ± 195	47
Cerebellum	28.1 ± 4.2	1494 ± 502	33.4 ± 10.1	714 ± 342	52
Cortex	30.1 ± 8.8	1890 ± 262	42 ± 16	903 ± 31	52
Medulla	28.6 ± 9.9	2052 ± 227	22.9 ± 6.3	826 ± 439	60

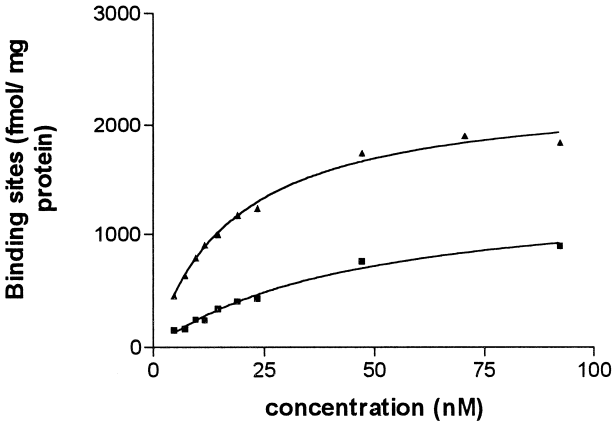

Fig. 3. Ligand concentration binding curves of [^{3}H](−)-vesamicol in the presence of 200 nM DTG (■) and in the absence of DTG (▲) in rat cortex membranes. Curve fitting yielded: Absence of DTG $K_d = 30.1$ nM, $B_{max} = 1890$ fmol/mg protein; Presence of 200 nM DTG $K_d = 42$ nM, $B_{max} = 903$ fmol/ mg protein. Goodness of fit: $R^2 = 0.99$ (absence of DTG), $R^2 = 0.98$ (presence of 200 nM DTG). Data displayed were obtained from three independent experiments. The proportion of binding sites occluded by DTG was 52%.

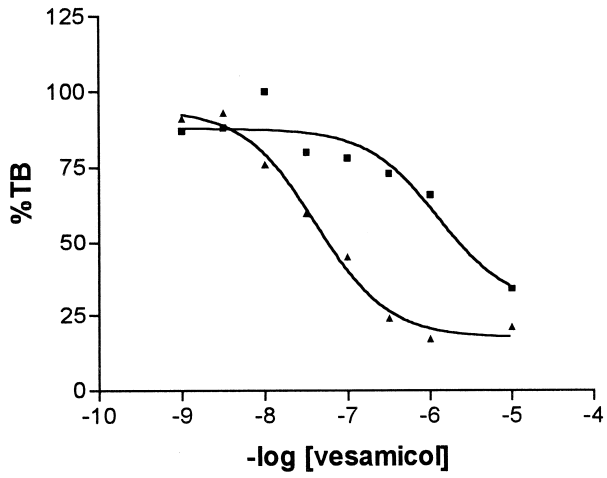

Fig. 4. Inhibition of [^{3}H](−)-vesamicol (4 nM) binding to rat striatum membranes by (+)-vesamicol (■) and (−)-vesamicol (▲) in the presence of 200 nM DTG. One-site curve fitting yielded: pIC_{50} (+)-vesamicol = 5.88, pIC_{50} (−)-vesamicol = 7.38. Goodness of fits: R^2 (+)-vesamicol = 0.91, R^2 (−)-vesamicol = 0.99. Data displayed were obtained from three independent experiments.

vesamicol derivatives (compounds **1**–**7**, see Fig. 1) we estimated the K_i values of these compounds in [^{3}H](−)-vesamicol binding assays with rat brain striatum, in the presence and the absence of DTG. In addition, the K_i values of these compounds for both the σ_1 binding site (using [^{3}H]haloperidol in guinea pig medulla oblongata) and the σ_2 binding site (using [^{3}H]DTG binding in fusion cells) were estimated. Compounds **4**–**7** were tested as racemic mixtures, whereas both enantiomers of compounds **1**–**3** were tested. The results are depicted in Table 2. These results demonstrate that vesamicol and the tested derivatives do not show a good selectivity for the acetylcholine transporter. All vesamicol derivatives (except vesamicol itself) displayed a higher affinity for the σ_1 binding site than for the acetylcholine transporter. Moreover, all compounds displayed a high σ_2 binding site affinity; however, the affinity for the σ_1 binding site was higher than that for the σ_2 binding site.

Fig. 4 illustrates the inhibition curve of (+)- and (−)-vesamicol binding (in the presence of 200 nM DTG) to the vesicular acetylcholine transporter in rat striatum. The observation that the (−)-isomer was approximately 30 × more potent than the (+)-isomer supports the specificity of this radioligand assay.

Since it has been reported that some of the tested vesamicol derivatives (the compounds (−)**1**, (+)**2**, (+)**3**,

Table 2
Inhibition parameters of specific [^{3}H](−)-vesamicol (4 nM) binding in the absence of DTG (assay 1) and in the presence of 200 nM DTG (assay 2) in rat striatum, [^{3}H]haloperidol binding in guinea pig medulla oblongata (σ_1) and [^{3}H]DTG binding in N18TG2 cells fused with septal neurones of the rat (σ_2)

Compound (see Fig. 1)	pIC_{50} (mean values ± S.D.) and K_i values (nM), calculated from the mean pIC_{50}							
	assay 1		assay 2		σ_1 site		σ_2 site	
	pIC_{50}	K_i (nM)	pIC_{50}	K_i (nM)	pIC_{50}	K_i (nM)	pIC_{50}	K_i (nM)
(−)1	7.54 ± 0.06	4.7	7.52 ± 0.03	26.6	6.99 ± 0.42	37.6	7.29 ± 0.14	42.3
(+)1	6.36 ± 0.41	374	6.05 ± 0.07	786	7.68 ± 0.23	7.7	7.34 ± 0.14	37.7
(+)2	8.31 ± 0.19	4.2	8.50 ± 0.21	2.8	7.89 ± 0.13	4.7	7.58 ± 0.37	21.7
(−)2	7.25 ± 0.09	48.2	6.90 ± 0.30	111	8.53 ± 0.41	1.1	7.42 ± 0.29	31.4
(+)3	7.64 ± 0.31	19.6	7.86 ± 0.29	12.2	7.64 ± 0.29	8.4	7.31 ± 0.30	40.4
(−)3	6.68 ± 0.25	179	6.53 ± 0.15	260	7.97 ± 0.24	3.9	7.25 ± 0.46	46.4
4	7.95 ± 0.16	9.6	8.12 ± 0.21	6.7	8.44 ± 0.07	1.3	7.47 ± 0.13	28.0
5	7.60 ± 0.11	21.5	7.50 ± 0.26	27.9	7.74 ± 0.08	6.7	7.24 ± 0.12	47.5
6	7.30 ± 0.04	42.9	7.90 ± 0.20	11.1	8.10 ± 0.10	2.9	7.37 ± 0.18	35.2
7	7.55 ± 0.07	24.1	7.95 ± 0.49	9.9	8.55 ± 0.38	1.0	8.14 ± 0.15	6.0
Haloperidol	6.73 ± 0.28	161	6.15 ± 0.33	624	8.43 ± 0.15	1.4	7.62 ± 0.08	19.8
DTG	5.69 ± 0.30	1749	—	—	7.30 ± 0.15	18.4	7.72 ± 0.08	16.4

Data displayed were obtained from three independent experiments. For calculation of K_i values, the following K_d values of the radioligands were used: assay 1, $K_d = 23.9$ nM; assay 2, $K_d = 29.9$ nM; σ_1, $K_d = 0.58$ nM (DeLoore et al., 1994); σ_2, $K_d = 18.9$ nM (DeLoore et al., 1994).

Table 3
Inhibition parameters of specific [^{3}H](−)-vesamicol binding in rat cortex determined with a two-site competition fit and a one-site competition fit (Graphpad Prism). Data displayed were obtained from two independent experiments. All inhibition curves were better described by a two-site competition binding curve ($P < 0.05$) by performing F-tests, except compound **4**, which was described better by a one-site competition binding curve

Compound (see Fig. 1)	Two-site competition fit		One-site competition fit	F values
	pIC_{50},1	pIC_{50},2	pIC_{50}	
(+)**2**	8.41	8.67	7.97	$F(2,11) = 5.37,\ P < 0.05$
(−)**2**	8.45	6.63	8.03	$F(2,13) = 11.73,\ P < 0.01$
4	8.35	7.39	8.14	$F(2,13) = 1.84,\ P > 0.05$
6	8.10	6.90	7.78	$F(2,13) = 4.11,\ P < 0.05$

4 and **5**) are able to discriminate between σ binding sites and the acetylcholine transporter (Efange et al., 1995), we performed an additional experiment. We assessed, by making detailed concentration curves, the inhibition of [^{3}H](−)-vesamicol by compounds (+)**2**, (−)**2**, **4** and **6**; (Table 3) in rat cortex. If the compounds were as discriminative as reported, one would expect a good correlation with the fit to a two-site competition curve, with large differences between the K_i values for the low- and high-affinity sites. Although curve fitting showed that the competition binding curves for the compounds (+)**2**, (−)**2** and **6** were better described by a two-site model and compound **4** by a one-site model, the binding parameters (see Table 3) were in agreement with our findings as presented in Table 2, which indicates a lack of selectivity of these compounds for the vesicular acetylcholine transporter.

4. Discussion

Our data clearly confirm the presence of stereoselective, high-affinity, binding sites of [^{3}H](−)-vesamicol in membrane preparations of different rat brain regions, suggesting that there are significant amounts of vesicular acetylcholine transporters in these brain regions. However, it is clear from our data that, in addition to binding to the vesicular acetylcholine transporter, vesamicol binds considerably to σ binding sites in rat brain tissue. This was demonstrated by using the selective σ-ligand DTG in combination with [^{3}H](−)-vesamicol. The inhibition of [^{3}H](−)-vesamicol binding by DTG, as visualized for instance in rat striatal tissue, correlated very well with binding to a two-site model. One site, with a K_i value in the low nanomolar range, reflects DTG binding to σ binding sites and the other, with a K_i value in the low micromolar range, reflects binding of DTG to the vesicular acetylcholine transporter. Based on the K_i values of DTG for both sites (21 ± 2 nM and 1.1 ± 0.1 μM, respectively), addition of 200 nM DTG in [^{3}H](−)-vesamicol binding experiments would be adequate to occlude all σ binding sites, whereas the vesicular acetylcholine transporter binding site would not be occupied at this concentration.

To acquire insight into the proportion of σ binding sites and acetylcholine transporter binding sites in different rat brain regions, a series of experiments was performed in the absence and the presence of 200 nM DTG. The reduction of [^{3}H](−)-vesamicol binding in the presence of 200 nM DTG was considerable in all brain regions studied and especially in the hippocampus, cortex, medulla oblongata and cerebellum, indicating significant numbers of σ binding sites in all these brain regions. These findings limit the usefulness of vesamicol as a selective cholinergic marker in these brain regions. This notion may explain the discrepancy between the loss of a smaller portion of [^{3}H](−)-vesamicol binding sites relative to the decrease in choline acetyltransferase activity in the neocortex and hippocampus following lesions of the septum in rats (Altar and Marien, 1988), in the amygdala of Alzheimer's disease patients (Kish et al., 1990) and in the temporal cortex of patients with Alzheimer's disease and Parkinson's disease (Ruberg et al., 1990).

In the second part of our study, we tested 7 vesamicol derivatives, including iodine-containing derivatives, as potential SPECT ligands, by determining their selectivity and affinity for the acetylcholine transporter in rat striatum and for σ binding sites (medulla oblongata tissue from guinea pigs and fusion cells for σ_1 and σ_2 binding sites respectively). The most potent vesamicol derivative in the acetylcholine transporter binding assay is (+)-MIBT {(+)**2**, Efange et al., 1993}. However, this compound displayed similar affinity for the σ_1 binding site in our hands. Also the other vesamicol derivatives we tested had a comparable or higher affinity for σ_1 binding sites than for the acetylcholine transporter. In addition they displayed a significant affinity for σ_2 binding sites. These findings seem to be in contrast to the findings reported by Efange et al., 1995. For some of the derivatives used in this study, they reported much higher K_i values (i.e., lower affinity) for both the σ_1 and σ_2 binding sites and much lower K_i values for the acetylcholine transporter binding, suggesting that these compounds could discriminate (by a factor of more than 100) between σ binding sites and the acetylcholine transporter. The differences in K_i values for the acetylcholine transporter found by us and Efange et al. might be explained by the use of different buffers and different animal species. We used rat brain tissue, whereas Efange et al. used synaptic vesicles from the electric organ of *Torpedo californica*. Although we both used guinea pig

brain for the σ_1 site binding, different radioligands were used ([^{3}H](+)-SKF 10,047 and [^{3}H]haloperidol respectively) and different brain regions (forebrain and medulla oblongata). In view of the possible existence of multiple σ site subtypes a discrepancy in findings may not be surprising.

Radioiodinated [^{125}I](−)-**7** has already been used in an in vitro characterization in rat cerebral membranes (Shiba et al., 1996). The conclusion was that this compound selectively labels vesicular acetylcholine transporters with an affinity which is comparable with that of (−)-vesamicol. The difference with our findings might be explained by the use of the racemic mixture of this compound, which means that only the (+)-isomer shows high σ site affinity.

Our findings were confirmed in an experiment in which we studied the inhibition of [^{3}H](−)-vesamicol binding in rat cortex by some of the vesamicol derivatives (compounds (+)**2**, (−)**2**, **4** and **6**). Using DTG inhibition, we clearly showed that, in the cortex, about 50% of the [^{3}H](−)-vesamicol binding reflects labelling of the acetylcholine transporter and 50% of the binding involves σ binding sites. Based on the reported values of Efange and coworkers, one would expect a clear two-site competition curve with large differences between the K_i values for the low- and high-affinity binding sites. In contrast, according to our findings one would expect a much smaller, if any, difference between the K_i values. This means for compound (+)**2** that with a two-site model the K_i values would be similar, which is indeed what we found. The results for the curve fitting for the other compounds are also in agreement with our results. Hence, all our findings point to the same conclusion, namely that vesamicol and the derivatives used in the present study are unable to discriminate between acetylcholine transporters and σ binding sites, at least in rat brain.

The poor selectivity of vesamicol and its derivatives limits their usefulness as selective markers for cholinergic nerve terminals, even in the striatum. To monitor the acetylcholine transporter, and by inference the integrity of the cholinergic system in the brain in vivo, one could perform SPECT studies using a radioactive iodine-containing vesamicol derivative in combination with a selective σ ligand that crosses the blood-brain barrier and occludes the σ binding sites. A better option might be, however, the development of iodine-containing vesamicol derivatives that really discriminate between acetylcholine transporters and σ binding sites and which have the highest affinity for the acetylcholine transporter. Alternatively, iodinated hemicholinium-3 derivatives, which display high affinity for the choline uptake carrier in the axonal membrane, could be developed.

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