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European Journal of Medicinal Chemistry 40 (2005) 1197–1205
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

Original article

Structural changes of benzylether derivatives of vesamicol and their influence on the binding selectivity to the vesicular acetylcholine transporter

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Received 3 May 2005; accepted 20 June 2005

Available online 10 August 2005

Abstract

^{18}F labelled vesamicol analogues, which bind to the vesicular acetylcholine transporter (VAChT) in central cholinergic nerve terminals, are expected to be potential radioligands for the visualisation of cholinergic transmission deficits via positron emission tomography (PET). In this report the regioselective synthesis of five novel vesamicol analogues as well as their in vitro binding properties to the VAChT are described. Beside having the 4-fluorobenzylether-substitution at the cyclohexyl ring as an unique structural feature, the new compounds are additionally modified at the phenyl and piperidine moiety of the vesamicol skeleton. The affinity and selectivity to the VAChT were analysed by competitive binding studies using tritium labelled radioligands. The VAChT affinities (K_i-values) of the novel compounds were estimated ranging between 7.8 ± 3.5 nM and 161.6 ± 17.3 nM, thus some of them are binding with higher affinity to the transporter than vesamicol. However, the compounds tested demonstrated also affinities to the sigma receptors σ_1 and σ_2 ranging between 4.1 ± 1.5 nM and 327.5 ± 75.9 nM. Nevertheless, these data provide the basis for future structure-binding-studies and further underline the potential and usefulness of vesamicol analogues for imaging of the VAChT.

Keywords: Vesamicol analogue; VAChT; PET; ^{18}F

1. Introduction

The cholinergic system in the brain is known to be involved in cognitive function and memory. Neurodegenerative disorders like Alzheimer's disease as well as physiological ageing processes are characterised by a profound loss of central cholinergic neurons which is accompanied by deficiencies in cholinergic neurotransmission [1–7]. The vesicular acetylcholine transporter (VAChT), localised in cholinergic nerve terminals [8,9], is responsible for the transport of acetylcholine into presynaptic vesicles [10,11]. Therefore, radioligands which bind specifically to the VAChT, are assumed to provide a tool for studying the density of central cholinergic neurons using PET (positron emission tomography) or SPECT (single photon emission computed tomography). Such substances afford an opportunity to investigate neurodegenerative processes in vivo and to follow up the efficiency of therapeutic strategies.

All of the previously described VAChT ligands are based on the structure of vesamicol, a drug that binds with high affinity to the vesamicol binding site of the transporter, whereby it non-competitively inhibits the transport of acetylcholine into the vesicles [12–15]. However, vesamicol demonstrates considerable affinity to sigma receptors [16], being also present in cholinergic brain regions, and which makes it

Abbreviations: VAChT, vesicular acetylcholine transporter; PET, positron emission tomography; DTG, 1,3-(di-*o*-tolyl)-guanidine.

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doi:10.1016/j.ejmech.2005.06.007

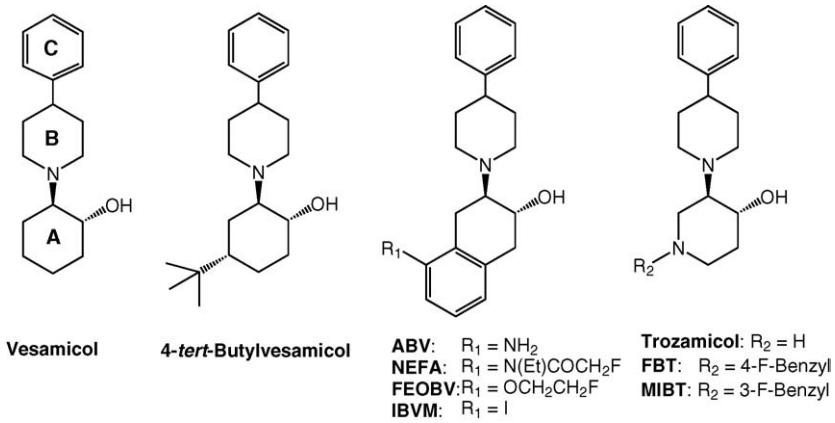

Fig. 1. Selected ligands for the vesicular acetylcholine transporter (VAChT).

less useful as a selective radiotracer for imaging cholinergic deficits. Therefore, in the past numerous efforts have been undertaken to improve the selectivity by chemical modifications of the vesamicol structure. A number of compounds are structurally derived from benzovesamicol including ABV [17], [^{18}F]NEFA [18], [^{18}F]FEOBV [19,20] or [^{125}I]IBVM [21,22] and from trozamicol e.g. [^{18}F]FBT [23–26] or [$^{123/125}$I]MIBT [27–29] (Fig. 1). Although most of them were described as promising radiotracers because of their in vivo evaluation in rats, rodents or nonhuman primates, only [^{123}I]IBVM and [^{18}F]NEFA were used for human studies [30–32]. But so far none of these ligands is really accepted for clinical application due to undesired side effects. For instance the extremely high lipophilicity of the iodinated molecule [^{123}I]IBVM causes a high accumulation of the compound in the intestine and liver which resulted in an unacceptable radiation exposure of the gastrointestinum.

With the aim to evaluate a potential ^{18}F labelled ligand, that binds with high affinity and selectivity to the VAChT, we developed the 4-O- and 5-O-substituted fluorobenzylether derivatives **1** and **2** (Fig. 2). In 2004, we reported on the synthesis and in vitro evaluation of this novel class of vesamicol analogues [33].

Especially the 4-O-fluorobenzylether derivative **1** was characterised by its high binding affinity to the VAChT (K_i = 10.7 ± 1.7 nM). However, it also exhibited a considerable affinity to both sigma receptors σ_1 and σ_2 (K_i = 18.5 ± 6.9 nM).

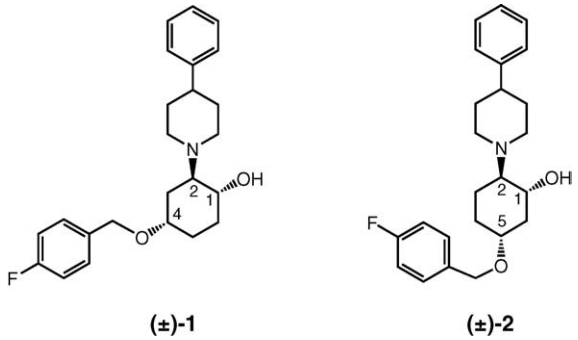

Fig. 2. 4-O- and 5-O-substituted fluorobenzylether derivatives **1** and **2**.

In this paper we report on selected structural modifications of **1** in order to decrease the affinity to sigma receptors, while the high binding affinity to VAChT is not affected. Substitutions on the ring B (piperidine moiety) and ring C (phenyl moiety) of the vesamicol skeletal structure resulted in the five new analogues **12a**,**b-15** (Fig. 3). To get reliable information on the binding properties of these derivatives, competitive in vitro binding assays were performed using rat brain tissue.

2. Chemistry

The new vesamicol analogues **12a**,**b-15** and their required key precursors, the amines **6a**,**b**, **7**, **10**, **11** were synthesised as outlined in Fig. 4 and Fig. 5.

The preparation of amines **6a** and **6b** (Fig. 4) based on procedures partially described in the literature [34–36]. Commercially available 1-benzyl-piperidine-4-one was reacted with p-fluorophenylmagnesium bromide in THF at 0 °C to give the tertiary alcohol **4a**. Elimination of water under acidic conditions delivered the olefine **5a**. Subsequent palladium catalysed hydrogenation by simultaneous debenzylation of **5a** in a one-step procedure yielded amine **6a**. The analogue bromo derivative **6b**, so far not described in the literature, was prepared by the same method using p-bromophenylmagnesium bromide as starting material.

The synthesis of amine **10** was already reported [37], but modifications of the known synthetic pathway resulted in an increased yield in our study. Thereby, **10** was prepared by methylation of 1-benzyl-piperidine-4-one using the commercially available Grignard reagent methylmagnesium chloride followed by Friedel-Crafts reaction with benzene and subsequent palladium catalysed debenzylation.

In general, the synthesised amines were converted into their oxalates.

4-Morpholinopiperidine **7** and 4-phenylpiperazine **11** for the synthesis of **14** and **15** are commercially available.

Applying a regioselective ring opening reaction of the *cis*-epoxide precursor **3** (Fig. 5) a convenient approach to the

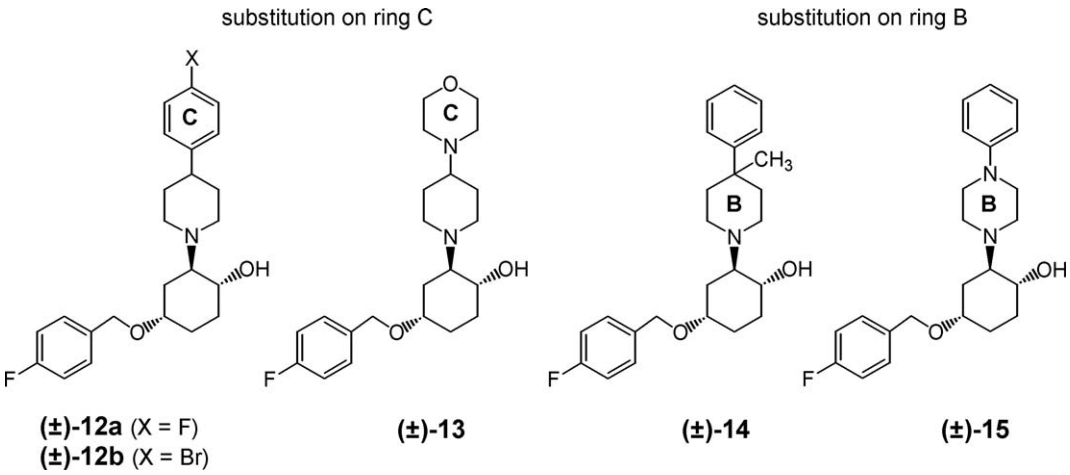

Fig. 3. Novel 4-O-fluorobenzylether derivatives of vesamicol.

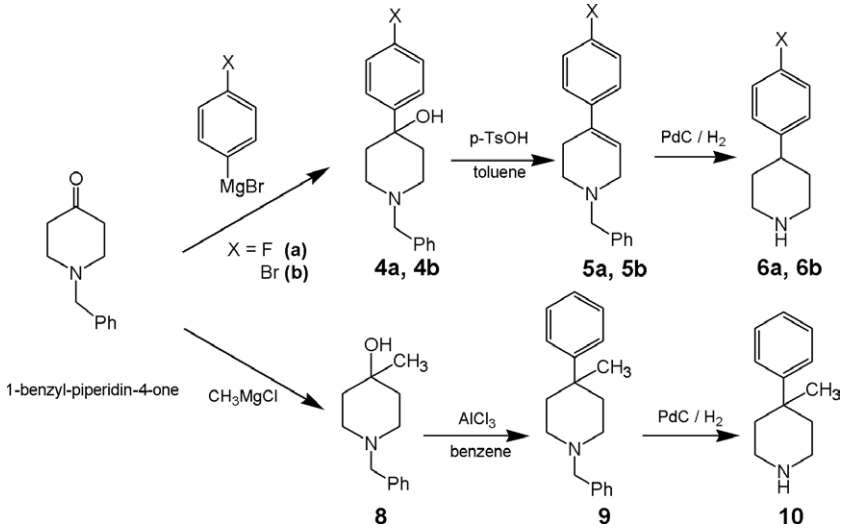

Fig. 4. Synthesis of amines **6a, 6b** and **10**.

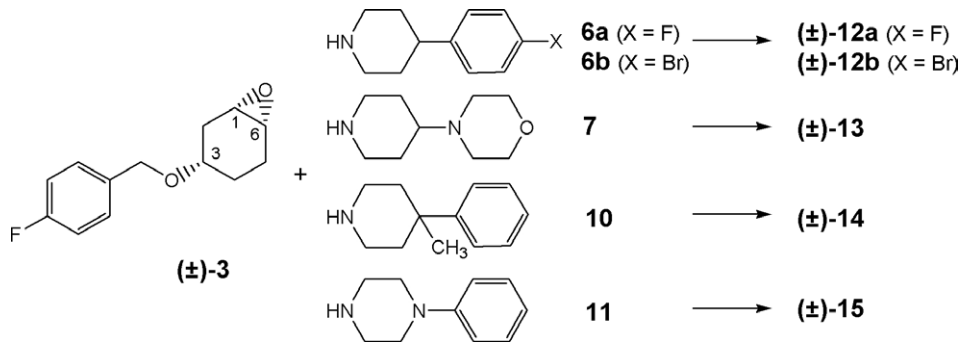

Fig. 5. Synthesis of **12a**,**b-15**.

4-substituted diasteriomerically pure aminoalcohols **12a**,**b-15** was achieved. Due to the favoured axial attack of a nucleophilic amine on C-1 of **3** only the 4-substituted regioisomeres are formed under normal reaction conditions [38]. This high regiochemical control was observed for all of the used amines **6a**, **6b**, **7**, **10** and **11**. In general, the ring opening reactions were carried out in ethanol at 70 °C under stirring for 4 days to provide crystalline products.

Cis-epoxide **3** was prepared according to methods described by Scheunemann et al. [33].

3. In vitro binding studies

To evaluate the binding affinity to VAChT, the fluorobenzylether derivatives **12a**,**b-15** (Fig. 3) were investigated in competitive binding assays on rat brain membranes using (-)-[^{3}H]vesamicol as high affinity VAChT radioligand (K_d = 23 nM, [39,40]). To determine the selectivity of binding to VAChT the compounds were investigated with regard to their affinity to sigma receptors. The sigma binding competition assays were performed using rat liver membranes which are known to be rich in σ_1 and σ_2 receptors. [^{3}H]DTG, one of the commonly used potent and selective sigma ligands (σ_1 and σ_2), was applied as radiotracer.

The potency of the new compounds to successfully compete with the binding of (-)-[^{3}H]vesamicol to the VAChT is expressed by their K_i-values determined according to the method of Cheng and Prussoff [41]. For comparison, (-)-vesamicol was analysed in the same assay run. The K_i-values measured in sigma receptor binding assays were compared to DTG as reference substance.

4. Results and discussion

This study continues our efforts to synthesise a highly specific ^{18}F labelled radioligand with the potency to visualise cholinergic dysfunction in vivo via the vesamicol binding site of the vesicular acetylcholine transporter in central neurons. In a previous work [33] we reported on the development of novel benzylether and fluorobenzylether derivatives of vesamicol either 4- or 5-substituted at the cyclohexyl ring on the basis of two different regioselective ring opening reactions. The 4-substituted analogues showed significantly higher binding affinities to the VAChT than the corresponding 5-O-ether derivatives. However, all isomers were characterised by their low selectivity as shown by nanomolar binding affinities to sigma receptors. Maintaining the 4-O-fluorobenzylether structure in the cyclohexyl ring of the vesamicol skeleton it was of fundamental interest to modify the two ring systems B and C of **1** in order to investigate the influence of structural changes on the binding selectivity. Inspired by modification strategies described for ring B and C in the literature [16,42] on the one hand, and aiming at a well balanced lipophilicity of the 4-O-fluorobenzylether derivatives on the other hand, the following chemical modifications were made: a) the phenyl ring (ring C) was fluorinated (**12a**), brominated (**12b**) and replaced by a morpholine ring (**13**), respectively and b) into the 4-position of the piperidine ring (ring B) a methyl group was introduced (**14**) and the piperidine ring was replaced by a piperazine ring (**15**) (Fig. 3).

Modifications of ring C (**12a**, **12b**, **13**):

Efange et al. reported in 1995 on vesamicol analogues concerning their sigma binding affinity [16]. Especially the conformationally restricted spirovesamicol showed a significant decrease in its binding affinity to sigma receptors when it was brominated on the phenyl group of the spiro ring system (adequate to the ring C fragment of vesamicol). Based on this result it was the aim to investigate whether the halogenation of the *para*-position of the phenyl group of **1** effects also a reduction of the sigma binding. Fluorination of **1** to get **12a** resulted in a decreased binding affinity to VAChT (K_i = 36.8 ± 2.4 nM) compared to **1** (K_i = 10.7 ± 1.7 nM), whilst the affinity to sigma receptors was unaffected. On the other hand, the bromination of **1** did not impair the binding affinity to VAChT, which is reflected by comparable K_i-values of **12b** and **1**. In contrast to the results of Efange et al. [16] who reported a decreased sigma receptor binding for the brominated spirovesamicol, the sigma affinity of **12b** increased remarkably (K_i = 4.1 ± 1.5 nM) compared to vesamicol (K_i = 183.4 ± 11.4 nM) and also marginal compared to **1**. Apparently, the introduction of a halogen atom into the *para*-position of the phenyl ring of the 4-fluorobenzylether derivative **1** led to a significant decrease of the selectivity factor K_{iDTG}/K_{iVes} = 0.5 for **12a** and **12b** compared to K_{iDTG}/K_{iVes} = 4.8 for vesamicol and also compared to K_{iDTG}/K_{iVes} = 1.7 for **1**.

In compound **13**, the phenyl ring was completely replaced by a morpholine ring, which is characterised by two hydrophilic hetero atoms and additionally by the chair conformation of the six membered ring compared to the planar aromatic phenyl group of **1**. It was the aim to study the influence of this completely different structural moiety on the one hand, and on the other hand to lower the lipophilicity of such compounds. However, the in vitro binding data showed clearly that these strong structural and electronic changes in the ring C fragment resulted in a dramatic decrease of the binding affinity of **13** to the VAChT (K_i about 9 000 nM). Rogers et al. [44] could show that the substitution of the phenyl ring in the vesamicol structure by a cyclohexyl ring, which has the same conformation as a morpholine ring, did not influence the capability of this ligand to inhibit the vesicular transport of [^{3}H]acetylcholine (the activity to block the acetylcholine storage of most of the known VAChT ligands is comparable to their affinity to the allosteric vesamicol binding site). Therefore it has to be assumed that the hydrophilic hetero atoms are responsible for the decreased binding affinity of **13** to VAChT. For instance multiple hydrogen bonds may cause this alteration of binding properties.

Modifications of ring B (**14** and **15**):

The replacement of the piperidine ring by a piperazine ring in compound **15** is based on the results of Bando et al. [42]. The piperazine analogue of iodobenzovesamicol (DRC140) has been shown to combine a high affine binding to the VAChT with a low affine binding to sigma receptors. In our study the introduction of the piperazine ring in **1** caused also a significant decrease of the sigma binding affinity (K_i = 327.5 ± 75.9 nM) compared to **1** (K_i = 18.5 ± 6.9 nM) and vesamicol (K_i = 183.4 ± 11.4 nM). However, this finding was accompanied by an unacceptable reduction of binding to the transporter (K_i = 161.6 ± 17.3 nM).

Table 1
Inhibition constants (K_i) of (-)-vesamicol, **1**, **12a**, **12b**, **13**, **14** and **15** for their binding to VAChT sites and to sigma receptor sites estimated from competition experiments in rat brain and rat liver membranes, respectively

Compound	VAChT ((-)-[^{3}H]vesamicol)[a]		σ_1, σ_2 ([^{3}H]DTG)[b]		Selectivity factor (K_{iDTG}/K_{iVes})
	K_i (nM)	N[c]	K_i (nM)	N[c]	
(-)-vesamicol	38.5 ± 4.2	12	183.4 ± 11.4	2	4.8
1	10.7 ± 1.7	6	18.5 ± 6.9	3	1.7
12a	36.8 ± 2.4	4	19.3 ± 1.5	6	0.5
12b	7.8 ± 3.5	4	4.1 ± 1.5	3	0.5
13	8 900	2	ND[d]	ND	ND
14	158.2 ± 37.1	4	57.1 ± 13.8	6	0.4
15	161.6 ± 17.3	6	327.5 ± 75.9	5	2.0

K_i-values were determined in competitive binding studies using different tritium labelled radioligands in the presence of the compounds tested at concentrations ranging from 10^{-11} M to 10^{-5} M. K_i-values are expressed as mean ± SD (nM).

[a] Binding to rat brain membranes, (-)-[^{3}H]vesamicol [6.1 ± 1.3 nM]; $K_{d\ rat\ brain}$ = 23 nM [39,40]. Nonspecific binding was defined as the binding of (-)-[^{3}H]vesamicol in the presence of 10^{-5} M unlabelled (-)-vesamicol hydrochloride.

[b] Binding to rat liver membranes, [^{3}H]DTG [4.9 ± 0.5 nM]; $K_{d\ rat\ liver}$ = 17.9 nM [43]. Nonspecific binding was defined as the binding of [^{3}H]DTG in the presence of 10^{-5} M unlabelled DTG.

[c] N, number of experiments performed.

[d] ND, not determined.

The introduction of a methyl group into the 4-position of the piperidine ring in compound **14** yielded in results contrary to those expected. Thus, a decrease in the binding affinity to the transporter (K_i = 158.2 ± 37.1 nM) was observed. The sigma binding affinity of **14** with a K_i = 57.1 ± 13.8 nM ranges between the K_i-values of **1** and vesamicol.

These findings indicate that chemical modifications of the 4-position of the piperidyl moiety result in structural changes which mainly impair the binding affinity to VAChT.

5. Conclusion

It was the aim of this study to evaluate novel structurally modified 4-O-fluorobenzylether derivatives of vesamicol with improved binding properties to the VAChT in comparison to compound **1** and to vesamicol itself, respectively. Both, vesamicol and **1**, were revealed to bind with high affinity but low selectivity to the transporter. The modifications of ring C and ring B fragments of the 4-fluorobenzylether derivative **1**, presented here, did not result in compounds potential and suitable for imaging cholinergic deficiencies as demonstrated by their in vitro binding data given in Table 1. Therefore, we suggest that the selectivity of vesamicol derivatives cannot be improved by chemical modifications of ring B or C. Nevertheless, the binding data obtained contribute to a better understanding of structural requirements for a selective binding to the VAChT and provide the basis for future structure-binding-studies.

6. Experimental

6.1. Chemistry

6.1.1. General

NMR spectra were recorded on Gemini 200 and Gemini 300 spectrometer and in one case on a Bruker DRX-600 (AVANCE). Melting points were determined using a Linström apparatus and are uncorrected. Mass spectra were recorded on a Mariner Biospectrometry workstation (Applied Biosystems) (MS-TOF) using direct inlet system and ElectronSpray Ionisation (ESI). Analytical TLC was performed on silica gel coated plates (Merck Kieselgel 60 F_{254}, 0.25 mm). The spots were identified using a UV lamp or by dipping into a solution of 2% ninhydrine in EtOH/MeOH 1:1 (especially for amines). Elemental analyses were performed in our laboratory using a CHN-*O*-Rapid analyser (Foss Heraeus) and agreed with the theoretical values within ± 0.4%.

Following abbreviations are used: EE for ethyl acetate, MTBE for methyl-*tert*-butylether, MeOH for methanol, EtOH for ethanol, THF for tetrahydrofuran, H for n-hexane.

Compounds **4a, 5a, 6a, 4b** and **5b** are described in the literature [34–36]. Since the authors used different reaction pathways or working up methods and most of these compounds are insufficient characterised the procedures and analytical data are listed again.

6.1.2. Synthetic procedures

6.1.2.1. 1-Benzyl-4-(4-fluoro-phenyl)-piperidin-4-ol (4a). To a mixture of a Grignard reagent prepared from magnesium turnings (2.20 g, 0.091 mol) and *p*-bromo-fluoro-benzene (10 mL, 0.091 mol) in 80 mL anhydrous THF a solution of 1-benzyl-piperidin-4-one (16.20 mL, 0.091 mol) in 20 mL THF was slowly added at 4 °C over a period of 120 min. The reaction mixture was stirred overnight and the resultant white slurry was hydrolysed using 4 M aqueous NH_4Cl (40 mL). The organic material was extracted with THF/MTBE (1:1) and dried over Na_2CO_3. Concentration of the solution yielded a brown oil, that was dissolved in 75 mL $CHCl_3$ followed by extraction of the basic products using aqueous citric acid 10% (3 × 30 mL). 6 M aqueous NaOH was added to get **4a** as free base and the organic material was re-extracted with $CHCl_3$ (4 × 40 mL). After drying over Na_2CO_3 the solution was concentrated to give 21.2 g (81%) of **4a** as orange oil. R_f = 0.38

(EE/H 1:1). ^{1}H NMR ($CDCl_3$, 200.14 MHz): δ (ppm) 7.46 (m, 2H, Ar), 7.32 (m, 2H, Ar), 7.01 (m, 2H, Ar), 3.58 (s, 2H, N-CH_2Ph), 2.74 (bm, 2H), 2.47 (bm, 3H), 2.13 (m, 2H) 1.74 (bm, 2H) (OH / Pip H2a, H2b, H3a, H3b). ^{13}C NMR ($CDCl_3$, 50.32 MHz): δ (ppm) 162.0 (d, $^1J_{CF}$ = 245.0 Hz), 144.5 (d, $^4J_{CF}$ = 3.0 Hz), 138.4, 129.4, 128.4, 127.3, 126.6 (d, $^3J_{CF}$ = 7.6 Hz), 115.0 (d, $^2J_{CF}$ = 21.1 Hz) (Ar), 71.2 (Pip C4), 63.4 (N-*C*H_2Ph), 49.6 (Pip C2), 38.7 (Pip C3).

6.1.2.2. 1-Benzyl-4-(4-bromo-phenyl)-piperidin-4-ol (4b). The synthesis of **4b** was carried out according to the same procedure described for **4a**. 19.80 g (83%) of **4b** was obtained as brownish oil. R_f = 0.50 (EE). ^{1}H NMR ($CDCl_3$, 199.97 MHz): δ (ppm) 7.44-7.28 (bm, 9H, Ar), 3.59 (s, 2H, N-CH_2Ph), 2.75 (bm, 2H), 2.45 (bm, 3H), 2.11 (m, 2H) 1.74 (bm, 2H) (OH / Pip H2a, H2b, H3a, H3b). ^{13}C NMR ($CDCl_3$, 50.28 MHz): δ (ppm) 171.1, 147.7, 138.2, 129.2, 128.2, 127.1, 126.6, 124.6 (Ar), 71.1 (Pip C4), 63.1 (N-*C*H_2Ph), 49.3 (Pip C2), 38.3 (Pip C3).

6.1.2.3. 1-Benzyl-4-(4-fluoro-phenyl)-1,2,3,6-tetrahydropyridine (5a). A solution of **4a** (5.20 g, 0.018 mol) and p-toluenesulfonic acid monohydrate (4.71 g, 0.025 mol) in 80 mL toluene was refluxed for 7 h using a dean stark trap to remove water. Concentration of the reaction mixture provides a brown residue, that was treated with 6 M aqueous NaOH. Extraction using $CHCl_3$, drying over Na_2CO_3 and concentration yielded a brown oil, that was purified via column chromatography (silica gel, EE/H 1:1) to get 2.91 g (62%) of **5a** as yellow oil. R_f = 0.71 (EE/H 1:1). ^{1}H NMR ($CDCl_3$, 200.14 MHz): δ (ppm) 7.47-7.20 (overlapping m, 7H, Ar), 7.03 (m, 2H, Ar), 6.04 (bs, 1H, Py H5), 3.68 (s, 2H, N-CH_2Ph), 3.20 (bm, 2H, Py H6), 2.75 (m, 2H, Py H2), 2.56 (bm, 2H, Py H3). ^{13}C NMR ($CDCl_3$, 50.33 MHz): δ (ppm) 162.2 (d, $^1J_{CF}$ = 245.6 Hz), 138.5, 137.2 (d, $^4J_{CF}$ = 3.8 Hz), 134.2, 129.4, 128.5, 127.4, 126.6 (d, $^3J_{CF}$ = 8.5 Hz), 122.1, 115.3 (d, $^2J_{CF}$ = 20.5 Hz) (Ar, Py C4), 65.4 (N-*C*H_2Ph), 53.5 and 50.1 (Py C2, C6), 28.4 (Py C3).

6.1.2.4. 1-Benzyl-4-(4-bromo-phenyl)-1,2,3,6-tetrahydropyridine (5b). **5b** was prepared using the same method described for **5a**. 4.49 g (0.013 mol) of **4b** and 3.38 g (0.018 mol) of p-toluenesulfonic acid monohydrate were used. The purification was carried out via column chromatography (silica gel, EE/H 3.5:1) to get 2.34 g (55%) of **5b** as orange oil. R_f = 0.87 (EE). ^{1}H NMR ($CDCl_3$, 199.97 MHz): δ (ppm) 7.45-7.22 (overlapping m, 9H, Ar), 6.07 (m, 1H, Py H5), 3.65 (s, 2H, N-CH_2Ph), 3.18 (bm, 2H, Py H6), 2.73 (m, 2H, Py H2), 2.54 (bm, 2H, Py H3). ^{13}C NMR ($CDCl_3$, 50.33 MHz): δ (ppm) 139.7, 137.9, 134.0, 131.4, 129.3, 128.3, 127.3, 126.5, 122.5, 119.8 (Ar, Py C4), 62.7 (N-*C*H_2Ph), 53.2 and 49.8 (Py C2, C6), 27.9 (Py C3).

6.1.2.5. 4-(4-Fluoro-phenyl)-piperidine (6a). To a solution of **5a** (1.74 g, 6.50 mmol) in 20 mL ethanol were added 2 mL acetic acid and 1.50 g PdC (10%). The flask was evacuated for a few seconds and filled with hydrogen. After stirring for 24 h at room temperature the catalyst was separated, the ethanolic solution was treated with aqueous 6 M NaOH and organic material was extracted using $CHCl_3$ (5 × 15 mL). Drying over Na_2CO_3 and concentration of the solution yielded in 0.90 g (77%) of nearly colourless oil. R_f = 0.73 ($CHCl_3$/MeOH/NH_3 2:1:0.2). ^{1}H NMR ($CDCl_3$, 300.06 MHz): δ (ppm) 7.20 (m, 2H, Ar), 7.01 (m, 2H, Ar), 3.22 (bm, 2H) and 2.76 (dt, 2H, J_{HH} = 12.3 Hz, J_{HH} = 2.2 Hz) (Pip H2a, H2b), 2.63 (tt, 1H, J_{HH} = 12.3 Hz, J_{HH} = 3.8 Hz, Pip H4), 1.86-1.82 (bm, 2H) and 1.64 (dq, 2H, J_{HH} = 12.8 Hz, J_{HH} = 3.6 Hz) (Pip H3a, H3b). ^{13}C NMR ($CDCl_3$, 50.28 MHz): δ (ppm) 161.4 (d, $^1J_{CF}$ = 243.9 Hz), 142.2 (d, $^4J_{CF}$ = 3.0 Hz), 128.1, (d, $^3J_{CF}$ = 7.5 Hz), 115.2 (d, $^2J_{CF}$ = 20.8 Hz) (Ar), 46.8 (Pip C2), 42.2 (Pip C4), 34.2 (Pip C3).

The amine was converted into its oxalate (M.p.: 158-160 °C) using an ethanolic solution of oxalic acid 10%.

6.1.2.6. 4-(4-Bromo-phenyl)-piperidine (6b). 0.81 g (80%) of **6b** was obtained as nearly colourless oil according to the same procedure described for **6a**. 1.36 g (4.16 mmol) of **5b**, 2 mL acetic acid and 1.50 g PdC were used. R_f = 0.80 ($CHCl_3$/MeOH/NH_3 2:1:0.2). ^{1}H NMR ($CDCl_3$, 300.06 MHz): δ (ppm) 7.23 (m, 3H, Ar), 7.30 (m, 2H, Ar), 3.39 (bd, 2H, J_{HH} = 12.6 Hz, Pip H2a), 2.91-2.82 (bm, 2H, Pip H2b), 2.68 (m, 1H, Pip H4), 1.95-1.89 (bm, 4H, Pip H3a, H3b), ^{13}C NMR ($CDCl_3$, 75.45 MHz): δ (ppm) 145.2, 128.6, 126.8, 126.6 (Ar), 45.9 (Pip C2), 41.9 (Pip C4), 32.3 (Pip C3).

A part of the product could be crystallised as free amine (M.p.: 144-146 °C). As described above the residual oil was converted into its oxalate (M.p. > 220 °C).

6.1.2.7. (±)-2-[4-(4-Fluoro-phenyl)-piperidin-1-yl]-4-(4-fluoro-benzyloxy)-cyclohexanol (12a). To a solution of epoxide **3** (0.66 g, 3.00 mmol) in 10 mL ethanol amine **6a** (0.4 g, 2.25 mmol) in 5 mL ethanol was added and stirred at 70 °C for 5 days. At 4 °C a mixture of **12a** and amine **6a** crystallised as white solid. The precipitate was purified via column chromatography (silica gel, EE to EE/MeOH 1:1) to give 0.45 g (55%) of nearly colourless ductile oil that partly crystallised from ethanol. M.p.: 91-92 °C. R_f = 0.41 (EE/H 1:1). ^{1}H NMR ($CDCl_3$, 200.14 MHz): δ (ppm) 7.32 (m, 2H, Ar), 7.17 (m, 2H, Ar), 7.00 (m, 4H, Ar), 4.48 (AB, 2H, J_{HH} = 12.0 Hz, O-CH_2PhF), 4.02 (bs, 1H, OH), 3.84 (bs, 1H, Cy H1), 3.47 (m, 1H, Cy H4), 2.95 (bd, 1H, Pip H2a), 2.84-2.64 (bm, 3H, Cy H2, Pip H6a, H6b), 2.48 (m, 1H, Pip H4), 2.27-1.25 (overlapping m, 11H, Pip H2b, H3a, H3b, H5a, H5b, Cy H3a, H3b, H5a, H5b, H6a, H6b). ^{13}C NMR ($CDCl_3$, 50.28 MHz, APT): δ (ppm) 162.2 (d, $^1J_{CF}$ = 244.8 Hz), 161.4 (d, $^1J_{CF}$ = 243.3 Hz), 141.9 (d, $^4J_{CF}$ = 3.0 Hz), 134.8 (d, $^4J_{CF}$ = 3.5 Hz), 129.0 (d, $^3J_{CF}$ = 8.0 Hz), 128.0 (d, $^3J_{CF}$ = 7.5 Hz), 115.2 (d, $^2J_{CF}$ = 21.6 Hz), 115.1 (d, $^2J_{CF}$ = 20.7 Hz) (Ar), 73.4 (Cy C1), 69.3 (O-*C*H_2PhF), 68.6 (Cy C4), 64.7 (Cy C2), 53.2 (Pip C6), 45.3 (Pip C2), 42.3

(Pip C4), 34.4 and 34.1 (Pip C3, C5), 27.7, 27.6, 26.3 (Cy C3, C5, C6). MS: *m/z* 402.2 (M+1). Anal. $C_{24}H_{29}F_2NO_2$ (C, H, N).

6.1.2.8. (±)-2-[4-(4-Bromo-phenyl)-piperidin-1-yl]-4-(4-fluoro-benzyloxy)-cyclohexanol (12b). To a solution of epoxide **3** (0.63 g, 2.85 mmol) in 10 mL ethanol amine **6b** (0.67 g, 2.85 mmol) in 5 mL ethanol was added and the reaction mixture was stirred at 70 °C for 5 days. At 4 °C a mixture of **12b** and amine **6b** crystallised as white solid. The precipitate was recrystallised from EtOH/$CHCl_3$ 1:3 to get 0.62 g (47%) of **12b** as white needles. M.p.: 121-122 °C. R_f = 0.33 (EE/H 1:2). ^{1}H NMR ($CDCl_3$, 199.97 MHz): δ (ppm) 7.29 (m, 6H, Ar), 7.04 (m, 2H, Ar), 4.47 (AB, 2H, J_{HH} = 12.0 Hz, O-C*H*$_2$PhF), 4.06 (bs, 1H, OH), 3.85 (bm, 1H, Cy H1), 3.47 (m, 1H, Cy H4), 2.95 (bd, 1H, Pip H2a), 2.85-2.63 (bm, 3H, Cy H2, Pip H6a, H6b), 2.48 (m, 1H, Pip H4), 2.27-1.24 (overlapping m, 11H, Pip H2b, H3a, H3b, H5a, H5b, Cy H3a, H3b, H5a, H5b, H6a, H6b). ^{13}C NMR ($CDCl_3$, 50.28 MHz): δ (ppm) 162.2 (d, $^1J_{CF}$ = 245.1 Hz), 146.3, 134.8 (d, $^4J_{CF}$ = 3.0 Hz), 129.0 (d, $^3J_{CF}$ = 8.1 Hz), 128.4, 126.8, 126.2, 115.2 (d, $^2J_{CF}$ = 21.5 Hz) (Ar), 73.5 (Cy C1), 69.2 (O-*C*H$_2$PhF), 68.6 (Cy C4), 64.6 (Cy C2), 53.3 (Pip C6), 45.3 (Pip C2), 43.1 (Pip C4), 34.3 and 34.0 (Pip C3, C5), 27.75, 27.70, 26.2 (Cy C3, C5, C6). MS: *m/z* 384.3 (M-Br+1). Anal. $C_{24}H_{29}BrFNO_2$ (C, H, N).

6.1.2.9. (±)-4-(4-Fluoro-benzyloxy)-2-(4-morpholin-4-yl-piperidin-1-yl)-cyclohexanol (13). A mixture of epoxide **3** (0.43 g, 1.80 mmol) in 10 mL ethanol and 4-morpholinopiperidine **7** (0.30 g, 1.70 mmol) in 5 mL ethanol was stirred at 70 °C for 5 days. The solution was concentrated to give a yellow oil, that was dissolved in 10 mL CH_2Cl_2 followed by extraction of the basic products using aqueous citric acid 10% (3 × 10 mL). 6 M aqueous NaOH was added to the water layer to get **13** as free base, that was re-extracted with CH_2Cl_2 (4 × 20 mL). After drying over Na_2CO_3 the solution was concentrated and the nearly colourless oily residue was purified via column chromatography (silica gel, EE/MeOH 1:1). After dissolving of the product in EtOH 0.32 g (34%) of **13** was obtained as white needles at 4 °C. M.p.: 128-129 °C. R_f = 0.54 ($CHCl_3$/MeOH/NH_3 10:1:0.1). ^{1}H NMR ($CDCl_3$, 600.13 MHz, HH Cosy): δ (ppm) 7.29 (m, 2H, Ar), 7.02 (m, 2H, Ar), 4.43 (AB, 2H, O-C*H*$_2$PhF), 3.92 (bs, 1H, OH), 3.80 (bm, 1H, Cy H1), 3.72 (m, 4H, Mor H3), 3.40 (dt, 1H, J_{HH} = 10.4 Hz, J_{HH} = 4.3 Hz, Cy H4), 2.87 (bd, 1H, Pip H2a), 2.71 (bm, 2H, Cy H2, Pip H6a), 2.55 (m, 5H, Mor H2, Pip H6b), 2.14 (bm, 1H, Pip H4), 2.06-2.01 (bm, 2H, Pip H2b, Cy H3a), 1.99-1.91 (bm, 2H, Cy H5a, H6a), 1.86 (bd, 2H, Pip H3a, H5a), 1.67 (bq, 1H), 1.34 (bt, 1H), 1.24 (dt, 1H, J_{HH} = 13.0 Hz, J_{HH} = 2.4 Hz) (Cy H3b, H5b, H6b), 1.56 (bq, 1H), 1.34 (bq, 1H) (Pip H3b, H5b). ^{13}C NMR ($CDCl_3$, 150.91 MHz, HETCOR, APT): δ (ppm) 162.2 (d, $^1J_{CF}$ = 245.3 Hz), 134.7, 129.0 (d, $^3J_{CF}$ = 8.0 Hz), 115.2 (d, $^2J_{CF}$ = 21,0 Hz) (Ar), 73.4 (Cy C1), 69.3 (O-*C*H$_2$PhF), 68.6 (Cy C4), 67.6 (Mor C3), 64.3 (Cy C2), 62.3 (Pip C4), 51.8 (Pip C6), 49.9 (Mor C2), 44.2 (Pip C2), 29.1 and 28.8 (Pip C3, C5), 27.69, 27.60, 26.3 (Cy C3, C5, C6). MS: *m/z* 393.2 (M+1). Anal. $C_{22}H_{33}FN_2O_3$ (C, H, N).

6.1.2.10. 1-Benzyl-4-methyl-piperidin-4-ol (8). To a solution of methylmagnesium chloride (20% in THF, 23 mL, 0.063 mol) at 4 °C under nitrogen a solution of 1-benzyl-piperidin-4-one (10 g, 0.053 mol) in 75 mL anhydrous THF was added. The reaction mixture was stirred overnight at room temperature, 4 M aqueous NH_4Cl (30 mL) was added and organic material was extracted with MTBE (3 × 15 mL). The combined organic solutions were dried over $MgSO_4$ and concentrated to yield 10.40 g (96%) of yellow oil. R_f = 0.15 (EE/H 1:1). ^{1}H and ^{13}C NMR spectra are in agreement with the literature [37].

6.1.2.11. 1-Benzyl-4-methyl-4-phenyl-piperidine (9). The reaction procedure was adopted from literature [37] and the analytical data are in agreement with the data reported.

6.1.2.12. 4-Methyl-4-phenyl-piperidine (10). To a solution of **9** (3.75 g, 0.014 mol) in 30 mL ethanol were added 2.5 mL acetic acid and 1 g PdC. The flask was evacuated for a few seconds and filled with hydrogen. After stirring for 24 h at room temperature the reduction was finished and the catalyst was separated. The ethanolic solution was treated with 6 M aqueous NaOH and organic material was extracted using $CHCl_3$ (5 × 15 mL) . Drying over Na_2CO_3 and concentration of the solution yielded 2.11 g (85%) of nearly colourless oil. By dissolving of the oily product in MTBE a part of compound **10** could be isolated as a white powder at 4 °C. M.p.: 69-72 °C. R_f = 0.69 ($CHCl_3$/MeOH/NH_3 2:1:0.2). ^{1}H and ^{13}C NMR spectra are in agreement with the literature [37].

For storage the rest of the amine was converted into its oxalate (M.p.: 180-185 °C) using an ethanolic solution of oxalic acid 10%.

6.1.2.13. (±)-4-(4-Fluoro-benzyloxy)-2-(4-methyl-4-phenyl-piperidin-1-yl)-cyclohexanol (14). To a solution of the epoxide **3** (0.47 g, 2.10 mmol) in 10 mL ethanol was added amine **10** (0.37 g, 2.10 mmol) in 5 mL ethanol. The reaction mixture was stirred at 70 °C for 5 days. After cooling the solvent was evaporated and the residue was dissolved in $CHCl_3$ (15 mL). After extracting of the basic products with 10% citric acid (4 × 5 mL), to the combined aqueous phases 6 M aqueous NaOH was added and organic material was re-extracted with $CHCl_3$ (4 × 15 mL). Following drying over Na_2CO_3 and evaporation of the solvent yielded an orange ductile oil, which was purified by column chromatography (silica gel, EE to EE/MeOH 1:1) to give 0.23 g (28%) of nearly colourless oil. R_f = 0.55 (EE). ^{1}H NMR ($CDCl_3$, 199.97 MHz): δ (ppm) 7.37-7.21 (m, 7H, Ar), 7.07 (m, 2H, Ar), 4.44 (AB, 2H, J_{HH} = 12.1 Hz, O-C*H*$_2$PhF), 3.80 (bm, 1H, Cy H1), 3.45 (m, 1H, Cy H4), 2.83-2.63 (overlapping m, 3H), 2.53-2.31 (bm, 2H), 2.24-1.57 (overlapping m, 8H), 1.28 (m, 2H) (Cy, Pip), 1.25 (s, 3H, C*H*$_3$). ^{13}C NMR ($CDCl_3$, 50.28 MHz, APT): δ

(ppm) 162.1 (d, $^{1}J_{CF}$ = 245.4 Hz), 149.1, 134.7 (d, $^{4}J_{CF}$ = 3.0 Hz), 129.0 (d, $^{3}J_{CF}$ = 8.1 Hz), 128.4, 125.7, 125.6, 115.1 (d, $^{2}J_{CF}$ = 21.2 Hz) (Ar), 73.4 (Cy C1), 69.2 (O-*C*H_2PhF), 68.5 (Cy C4), 64.4 (Cy C2), 45.3 and 45.0 (Pip C2, C6), 37.7 and 37.6 (Pip C3, C5), 36.5 (*C*H_3), 29.4 (Pip C4), 27.7, 27.6, 26.2 (Cy C3, C5, C6). MS: *m/z* 398.3 (M+1). To get **14** as a solid the oily product was converted into its oxalate using an ethanolic solution of oxalic acid 10%. M.p.: 214-215 °C. Anal. $C_{27}H_{34}FNO_6$ (C, H, N).

6.1.2.14. (±)-4-(4-Fluoro-benzyloxy)-2-(4-phenyl-piperazin-1-yl)-cyclohexanol (15). A mixture of epoxide **3** (0.75 g, 3.37 mmol) in 10 mL ethanol and 4-phenylpiperazine **11** (0.36 g, 2.25 mmol) in 5 mL ethanol was stirred at 70 °C for 5 days. The solution was concentrated to give a brown oil, that was dissolved in 10 mL CH_2Cl_2 followed by extraction of the basic products using aqueous citric acid 10% (4 × 10 mL). 6 M aqueous NaOH was added to the water layer to get **15** as free base that was re-extracted with CH_2Cl_2 (3 × 20 mL). After drying over Na_2CO_3 the solution was concentrated and the nearly colourless oily residue was dissolved in EtOH. At 4 °C 0.43 g (49%) of **15** was obtained as white needles. M.p.: 105-106 °C. R_f = 0.73 (EE). ^{1}H NMR ($CDCl_3$, 199.97 MHz): δ (ppm) 7.32 (m, 4H, Ar), 7.05-6.84 (m, 5H, Ar), 4.47 (AB, 2H, O-C*H*$_2$PhF), 3.92 (s, 1H, OH), 3.85 (bs, 1H, Cy H1), 3.52 (dt, 1H, J_{HH} = 10.4 Hz, J_{HH} = 4.4 Hz, Cy H4), 3.30-3.12 (bm, 4H, Pip H3a, H3b), 2.92-2.75 (bm, 3H, Pip H2a, Cy H2), 2.62-2.51 (bm, 2H, Pip H2b), 2.16-1.97 (bm, 3H), 1.77 (m, 1H), 1.35 (m, 2H) (Cy H3a, H3b, H5a, H5b, H6a, H6b). ^{13}C NMR ($CDCl_3$, 50.28 MHz, HETCOR): δ (ppm) 167.1 (d, $^{1}J_{CF}$ = 245.2 Hz), 151.3, 134.7 ($^{4}J_{CF}$ = 3,1 Hz), 129.1 (d, $^{3}J_{CF}$ = 8.1 Hz), 128.7, 119.9, 116.2, 115.2 (d, $^{2}J_{CF}$ = 21,3 Hz) (Ar), 73.4 (Cy C1), 69.3 (O-*C*H_2PhF), 68.5 (Cy C4), 64.3 (Cy C2), 49.9 (Pip C3), 48.2 (Pip C2), 27.6, 27.5, 26.3 (Cy C3, C5, C6). MS: *m/z* 385.2 (M+1). Anal. $C_{23}H_{29}FN_2O_2$ (C, H, N).

6.2. Radioligand binding and competition experiments

6.2.1. Radioligands and drugs

(-)-[^{3}H]Vesamicol ([^{3}H]2-(4-phenylpiperidinyl)-cyclohexanol, AH 5183, specific radioactivity 1258 GBq/mmol resp. 34 Ci/mmol) and [^{3}H]DTG ([^{3}H]1,3-(di-*o*-tolyl)-guanidine, specific radioactivity 1110 GBq/mmol resp. 30 Ci/mmol) were obtained from Perkin Elmer Life Sciences, Boston, MA, USA). (-)-Vesamicol hydrochloride and 1,3-(di-*o*-tolyl)-guanidine (DTG) were purchased from TOCRIS, BIOTREND Chemikalien GmbH, Köln.

6.2.2. Tissue preparation

Female Sprague Dawley rats (150–200 g) were anaesthetised with ether and decapitated. Their brains were rapidly removed from the skull. The brain tissue minus cerebellum was homogenised in 10 volumes (w/v) of ice-cold 0.25 M sucrose solution using a Teflon-glass homogeniser (50 strokes). Aliquotes were stored at -20 °C until use. Crude membranes for drug competition assays were obtained by centrifugation of the brain homogenate at 15 000 x g for 15 min at 4 °C, washed twice (once with 0.25 M sucrose solution, once with distilled water) and re-suspended to a concentration of 200 mg/mL wet tissue in assay buffer (50 mM Tris pH 7.4 containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$).

Rat liver (for analysing sigma receptor binding) was prepared in the same way. The liver lobes were minced before homogenisation.

6.2.3. Determination of binding data

Various concentrations (10^{-11} to 10^{-5} M) of the synthesised compounds were incubated in duplicates with membranes (600 µg protein) for 2 h at 25 °C during agitation (200 U min^{-1}) in a total volume of 1 mL of assay buffer in the presence of one of the different radiotracers: (-)-[^{3}H]vesamicol (AH 5183) [6.1±1.3 nM] and [^{3}H]DTG (σ_1 and σ_2 ligand, [4.9 ± 0.5 nM]), respectively. Unlabelled (-)-vesamicol and DTG were tested for comparison in parallel experiments. After incubation membrane bound radiotracer was separated from free radioligand by rapid vacuum filtration through Whatman GF/B glas-fiber filters presoaked for 3 h in 0.5% polyethyleneimine using a Brandel cell harvester (Gaithersburg, MD). The filters were washed 3 times with 4 mL ice-cold buffer - dried, soaked in 10 mL Rotiszint eco plus cocktail overnight and counted in a TriCarb Liquid Scintillation Counter (Tri-Carb2900TR, Packard, Meriden, USA) at 70% counting efficiency.

6.2.4. Data analysis

Inhibitory concentration at 50% specific binding of the radioligand (IC_{50}-value) was determined by a nonlinear curve fitting method, using Graphpad Prism, Vers. 3 (GraphPad Software, Inc., San Diego, USA). Specific binding of radioligands was defined as total binding minus nonspecific binding. Nonspecific binding was determined in parallel experiments in the presence of 10^{-5} M of the corresponding unlabelled ligand. K_i-values were derived from IC_{50}-values according to the equation: $K_i = IC_{50}/(1+C/K_d)$ [41] whereby C is the concentration of the radioligand and K_d is the dissociation constant of the radioligand. K_d-values were taken from the literature as indicated.

Acknowledgments

This work has been supported by a grant from Sächsisches Ministerium für Wissenschaft und Kunst, contract no. 7531.50-03-0361-01/6.

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