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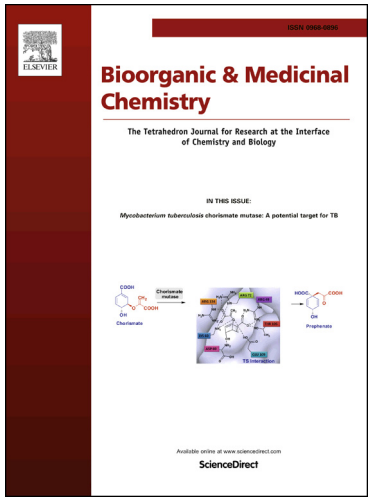

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Do spiroindolines have the potential to replace vesamicol as lead compound for the development of radioligands targeting the vesicular acetylcholine transporter?

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Abstract

The vesicular acetylcholine transporter (VAChT) is an important target for *in vivo* imaging of neurodegenerative processes using positron-emission-tomography (PET). So far the development of VAChT PET radioligands is based on the single known lead compound vesamicol. In this study we investigated a recently published spiroindoline based compound class (Sluder et al. 2012), which was suggested to have potential in the development of VAChT ligands. Therefore, we synthesized a small series of *N*,*N*-substituted spiro[indoline-3,4'-piperidine] derivatives and determined their *in vitro* binding affinities toward the VAChT. In order to investigate the selectivity, the off-target binding toward σ_1 and σ_2 receptors were determined. The compounds possessed VAChT affinities with K_i values in the range of 39 to 376 nM. Binding affinities toward the σ_1 and σ_2 receptors are in a similar range indicating that the strong structural difference between the spiroindolines and vesamicol did not improve the selectivity. The observed potential to additionally bind to σ receptors let us assume that the herein investigated spiroindolines are not suitable to replace vesamicol as lead compound for the development of VAChT ligands.

Keywords: VAChT, Vesamicol, Spiroindolines, PET, sigma receptors

1. Introduction

The vesicular acetylcholine transporter (VAChT) is a transmembrane protein located on presynaptic vesicles of cholinergic neurons. It transports the neurotransmitter acetylcholine into the vesicles for storage and controlled release and thus is an essential part of cholinergic neurotransmission.[1, 2] Exclusively expressed in the presynaptic part of cholinergic neurons, the VAChT is considered as valuable target for the investigation of cholinergic degeneration as a key feature related to the cognitive decline in Alzheimer's Disease (AD).[3-6] Noninvasive *in vivo* imaging based on radiolabeled VAChT ligands using PET (positron emission tomography) or SPECT (single-photon emission

computed tomography) is a promising option for visualization of such cholinergic neuronal processes and thus might support early diagnostics of AD.[7, 8]

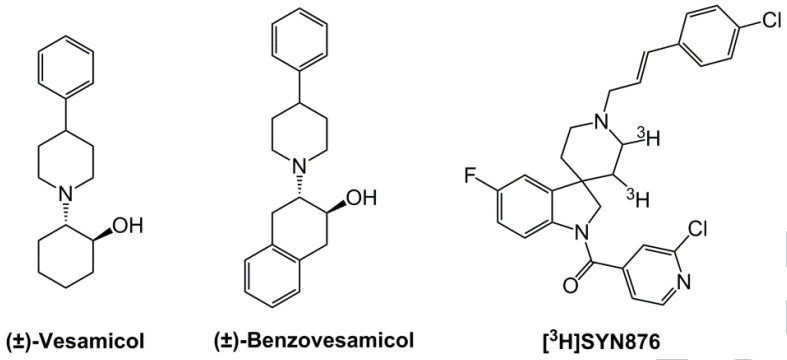
So far, the development of VAChT radiotracers is based on vesamicol (*trans*-2-(4-phenylpiperidino)cyclohexanol, Figure 1), the single known chemical lead compound, that binds with high affinity to an allosteric site of the transporter.[9, 10] However, radiolabeled vesamicol itself is not suitable for specific VAChT imaging due to its insufficient selectivity caused by the additional binding to the σ_1 and σ_2 receptors[11, 12], which are both as well expressed in cholinergic brain regions. Because so far only vesamicol-like structures are known to be tolerated by this allosteric binding site at VAChT, the development of selective VAChT ligands has focused mainly on structural modifications of vesamicol with the aim to keep or improve the affinity for VAChT and to reduce the affinity for sigma receptors. Several classes of vesamicol analogs have been studied such as benzovesamicols,[13-18] trozamicols,[19, 20] morpholino vesamicols,[21, 22] and azaspirovesamicols[23]. For some of these compounds moderate or high binding affinities to VAChT were determined *in vitro* and *in vivo.* In particular benzovesamicol derivatives labelled with fluorine-18 or iodine-123 have been used to investigate cholinergic pathways in humans by measuring VAChT expression via PET imaging.[14, 24, 25] However, despite promising results none of the ligands has been proven to be optimal for clinical application so far.

When studying the literature in detail, it turned out that the investigation of target selectivity of vesamicol analogs has been often neglected. To get a deeper insight into the structure-affinity *and* -selectivity relationship of vesamicol analogs, we recently synthesized a library of systematically modified derivatives and investigated them regarding their *in vitro* binding toward the VAChT and the off-target binding toward σ_1 and σ_2 receptors.[26] A main result of this study was the observation that an increase of VAChT affinity was often accompanied by an increase in σ_1 and/or σ_2 receptor affinity. Finally, our results demonstrated the challenges in the development of VAChT PET imaging ligands based on vesamicol as lead compound.

In 2012, Sluder et al. reported on *N*,*N*-substituted spiro[indoline-3,4'-piperidine] derivatives[27] and the linkage of their toxic action to the VAChT in insects.[28] The authors demonstrated that the binding of the selected spiroindoline derivative [^{3}H]SYN876 (Figure 1) in insect tissue is related to a single binding site in rat PC12 cells transfected with the VAChT gene of *Drosophila melanogaster*. Accordingly, they proposed this compound class as alternative to vesamicol for the development of VAChT ligands for PET imaging of neurodegenerative diseases.

In order to examine the potential of these spiroindolines as a new lead compound class for the development of VAChT PET ligands, we i) synthesized selected derivatives of the reported series and developed new derivatives with slight structural modifications, ii) determined their *in vitro* binding

affinity toward VAChT as well as toward σ_1 and σ_2 receptors and iii) investigated the binding mechanism of SYN876 and vesamicol using rat PC12 cells transfected with rat VAChT.



(±)-Vesamicol **(±)-Benzovesamicol** **[^{3}H]SYN876**

Figure 1. Known VAChT ligands vesamicol, benzovesamicol and spiroindoline [^{3}H]SYN876 as potential lead compounds in the development of VAChT PET radiotracers

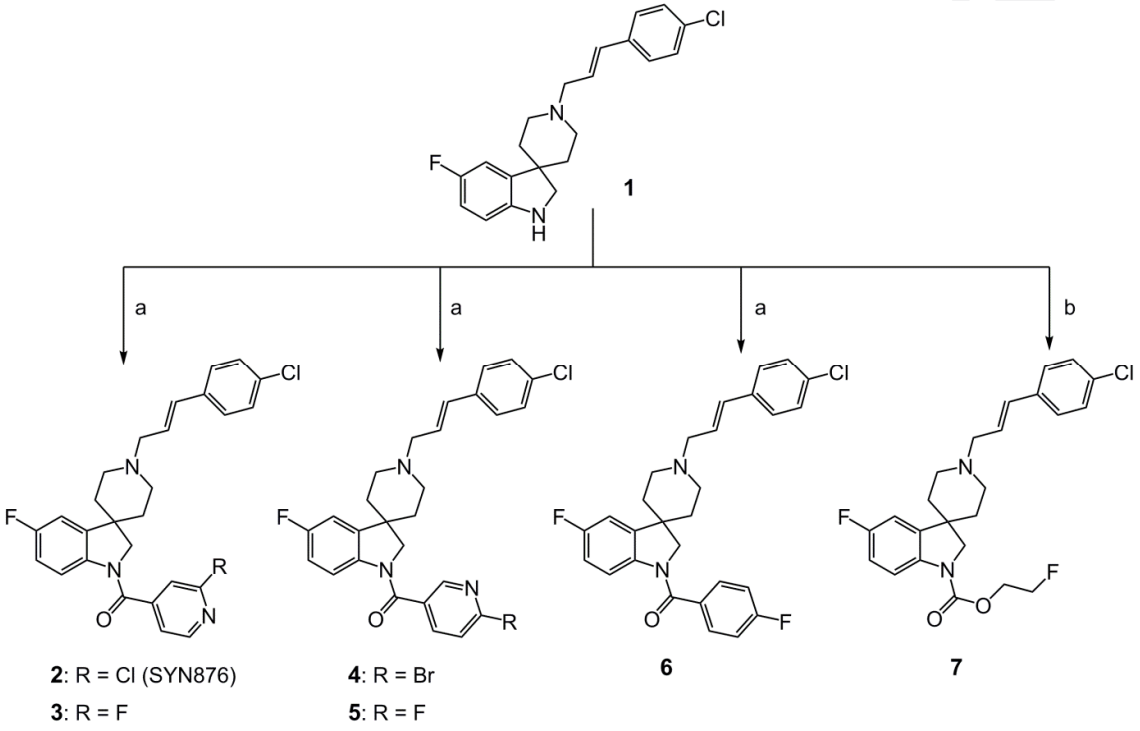
2. Results and discussion

In order to investigate the *in vitro* binding affinities of the *N*,*N*-substituted spiro[indoline-3,4'-piperidine] derivatives for the VAChT, we synthesized the already published[28] derivatives **2** (SYN876) and **3** (Scheme 1). With respect to the development of an ^{18}F-labelled PET radiotracer, we additionally designed four new compounds (**4**-**7**) bearing fluorine containing groups substituted at the indoline nitrogen. The *in vitro* binding affinities (K_i values) of all derivatives toward the VAChT and the σ_1 and σ_2 receptors were determined by radioligand displacement studies. Since vesamicol binds to an allosteric site at the vesicular acetylcholine transporter, we also investigated whether the binding site of compound **2** is identical to the vesamicol binding site.

2.1 Chemistry

The synthesis of the derivatives **2**-**7** was performed on the basis of the synthetic route reported by Sluder at al.[28] In a first step, compound **1** was synthesized according to the already in detail described procedure[28] (Scheme is given in the supplementary material). Briefly, 4-chlorocinnamyl chloride was coupled with 1,4-dioxa-8-azaspiro[4,5]decane and deprotected to the corresponding 4-piperidinone derivative. Subsequent Wittig reaction with methoxymethyltriphenylphosphonium chloride gave the corresponding methoxymethylenepiperidine which was allowed to react with 4-fluorophenylhydrazine and trifluoroacetic acid in a Fischer-Indole like reaction to give the spiroindoline derivative **1**. Compound **1** served as starting material to substitute different aroyl (**2**-**6**) groups at the secondary nitrogen of the indoline core by using triethylamine (TEA) and (benzotriazol-

1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (Scheme 1). For the synthesis of compound **7** we selected a simple and mild method for introducing the 2-fluoroethylester group. Thus, 2-fluoroethyl methanesulfonate and potassium carbonate as source for the carbonyl functionality reacted with the amine **1** to give the carbamate **7** in moderate yield. The formation of the corresponding *N*-alkylated product was not observed. Similar carbamation reactions of amines have been already described in literature and are beneficial in terms of handling when comparing to the common method of using alkyl chloroformates.[29, 30]



Scheme 1. Reagents and conditions: a) BOP, TEA, 2-chloroisonicotinic acid for **2**, 2-fluoroisonicotinic acid for **3**, 6-bromonicotinic acid for **4**, 6-fluoronicotinic acid for **5** and 4-fluorobenzoic acid for **6**, CH_2Cl_2, rt, 24 h; b) K_2CO_3, DMF, 2-fluoroethyl methanesulfonate, 90 °C, 19 h.

2.2 *In vitro* binding affinities

The compounds **2**-**7** were assessed regarding their affinities toward rat VAChT, rat σ_1 receptor and rat σ_2 receptor. The K_i values and experimental conditions are presented in Table 1.

Table 1. *In vitro* binding affinities of spiroindolines **2**-**7** and reference compounds (±)-vesamicol and (±)-benzovesamicol

Compd	K_i (VAChT)[a]	K_i (σ_1)[b]	K_i (σ_2)[c]
(±)-vesamicol[d]	47.4 ± 6.3	20.4 ± 4.7	112 ± 36

(±)-benzovesamicol[d]	1.32 ± 0.25	26.1 ± 4.5	96.4 ± 22.3
2	153 ± 49	137 ± 32	1185 ± 484
3	110 ± 38	55.4 ± 14.8	748 ± 229
4	39.2 ± 8.4	179 ± 14	60.1 ± 17.8
5	376 ± 95	282 ± 85	> 10000
6	243 ± 78	293 ± 47	1205 ± 128
7	328 ± 42	44.1 ± 7.6	1520 ± 169

K_i values in nM (mean ± S.D., n≥2) were derived from IC_{50} values according to the Cheng-Prusoff equation: $K_i = IC_{50}/(1+C/K_D)$, where C is the concentration of the radioligand and K_D the dissociation constant of the corresponding radioligand; [a] (-)-[^{3}H]vesamicol: K_D = 25.6 nM to VAChT on rVAChT-PC12 cells; [b] (+)-[^{3}H]pentazocine: K_D = 6.9 nM to σ_1 receptors on rat cortex homogenates; [c] [^{3}H]DTG with blocking of σ_1 receptors using 1 µM dextrallorphan: K_D = 29.0 nM to σ_2 receptors on rat liver homogenates; [d] K_i values were taken from Barthel et al.[26]

The bromonicotinyl substituted derivative **4** shows the highest affinity for the VAChT within the investigated spiroindoline series with a K_i value close to that of vesamicol (39.2 nM vs. 47.4 nM). Interestingly, the substitution of the bromine of **4** by a fluorine atom to achieve compound **5** caused an almost 10-fold reduction of the VAChT binding affinity. According to our data, the two published [28] derivatives **2** and **3** show only moderate binding to the rat VAChT with K_i values of 153 and 110 nM, respectively. In particular compound **2** was proposed by Sluder et al.[28] as alternative for the development of VAChT PET radioligands. However, when comparing the binding affinity of **2** with (±)-benzovesamicol (K_i(VAChT) = 1.32 nM), compound **2** is much less suitable because of a more than 100-fold lower VAChT affinity. The introduction of a fluorobenzoyl (**6**) and a fluoroethyl carboxylate (**7**) function at the indoline nitrogen did not improve the binding affinity for VAChT compared to **2** and **3**.

Surprisingly, the investigated *N*,*N*-substituted spiro[indoline-3,4'-piperidine] derivatives demonstrated certain affinity for the σ_1 receptor. The K_i values of compounds **2**-**7** for this target are in the range of about 45-300 nM. Moreover, derivatives **3** and **7** even show higher binding affinity for the σ_1 receptor than for the VAChT. This observed off-target binding has to be interpreted as a serious drawback of the compound class. Beside an improvement of the VAChT binding affinity additionally a lowering of the σ_1 receptor binding would have to be achieved when developing new VAChT ligands based on this spiroindoline scaffold.

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Generally, the binding affinities of the investigated derivatives **2**-**7** for the σ_2 receptor are considerably lower. Only compound **4** (K_i = 60.1 nM) shows a higher affinity for this target which impairs its selectivity for the VAChT.

2.3 Binding sites of compound 2 and vesamicol

Besides the orthosteric binding site of VAChT for the natural ligand acetylcholine, the transporter protein possess an allosteric binding site for the inhibitor vesamicol. To assess the potential of compound **2** and derivatives thereof for molecular imaging of VAChT, it is crucial to analyse in detail the mechanisms of interaction between the protein and the ligands. In a first attempt, we performed radioligand displacement studies to investigate, if compound **2** competes with (±)-vesamicol for the same binding site at VAChT.[31]

As shown in Figure 2, increasing concentrations of compound **2** (0 nM, 100 nM, 1000 nM) result in a rightward shift of the binding curves reflecting an increase in the apparent value of K_i of (±)-vesamicol. By linearization of this set of sigmoid curves according to Hanes-Woolf[32, 33] we obtained a pattern of almost parallel lines (Figure 3). Because such pattern can be taken as indicative for a competition mechanism[34], we assume that compound **2** and vesamicol bind to the same allosteric binding site of VAChT. This finding is quite interesting, because so far only vesamicol like compounds have been described to bind with comparatively high affinity to the VAChT. Obviously, the binding pocket is more flexible than expected, since the molecular structure of compound **2** is much different compared to vesamicol.

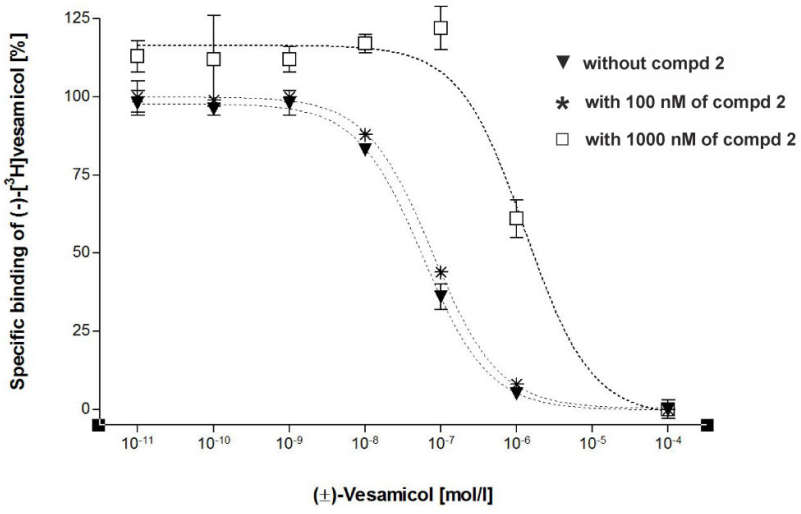

Figure 2. Representation of binding data as non-linearized inhibition curves (one out of three experiments; n=3 per data point).

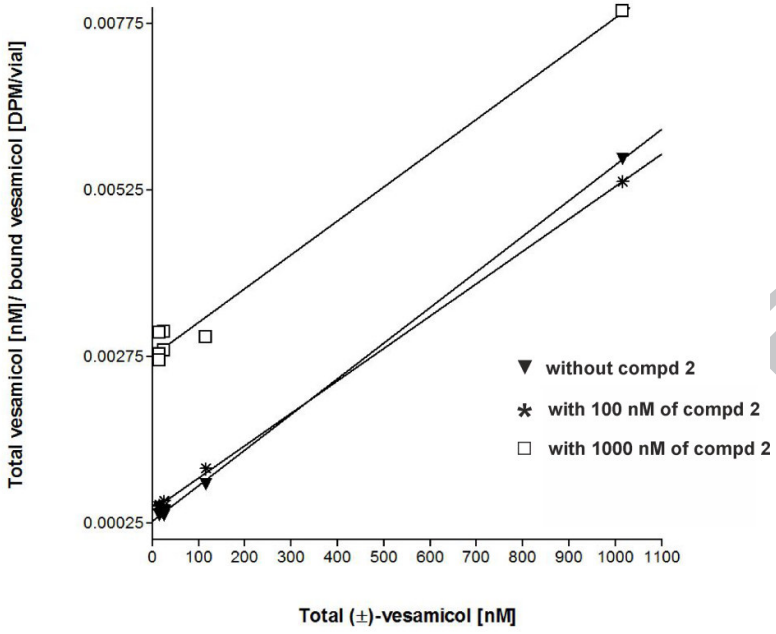

Figure 3. Representation of binding data after Hanes-Woolf linearization.

4. Conclusion

In order to investigate the potential of the recently published *N*,*N*-substituted spiro[indoline-3,4'-piperidine] derivatives[28] as new lead compounds for the development of PET radioligands for imaging of the vesicular acetylcholine transporter, we synthesized a small series of derivatives (**2**-**7**) and determined their *in vitro* binding affinities for the VAChT and the main off-target binding to σ_1 and σ_2 receptors. According to our data, all derivatives bind with moderate to low affinities toward the VAChT and show in addition considerable affinities for σ_1 and/or σ_2 receptors. Because of this general potential to bind to σ receptors, we conclude that the herein investigated spiroindolines are not suitable to replace vesamicol analogues as future lead compounds for the development of VAChT PET radioligands.

5. Material and methods

5.1. Chemistry

Analysis of all compounds was performed by HRMS, HPLC, TLC, and NMR spectroscopy. The spectra and chromatograms are given in the Supplementary material.

High resolution mass spectra were recorded on a ESI-qTOF Impact II (Bruker Daltonik GmbH) and on a ESI-TOF micrOTOF (Bruker Daltonik GmbH) using ElectroSpray Ionization (ESI). NMR spectra (^{1}H, ^{13}C,

^{13}C-APT, ^{19}F, H,H-COSY, HSQC, HMBC) were recorded on spectrometers from Varian. Splitting patterns have been designated as follows: s: singlet, d: doublet, bs: broad singlet, m: multiplet, t: triplet, dd: doublet of doublet, td: triplet of doublet; dt: doublet of triplet.

Analytical thin-layer chromatography (TLC) was performed on silica gel coated plates (Machery-Nagel, ALUGRAM SIL G/UV$_{254}$). The spots were identified using a UV lamp or by spraying a solution of 0.1% ninhydrine in EtOH/H_2O 1:10 or dipping into a $KMnO_4$-solution (3.0 g $KMnO_4$, 20 g K_2CO_3, 0.25 mL glacial acid, 300 mL water).

For purification of final products flash column chromatography was used with silica gel ZEOsorb 60 / 40-63 microns from Apollo Scientific Ltd.

The chemical purity of all compounds is ≥ 97% and was controlled by HPLC using a 150 × 3 mm Reprosil-Pur Basic HD - 3 µm column (Dr. Maisch GmbH, Germany). These analytical chromatographic separations were performed on a Dionex Ultimate 3000 system, incorporating a LPG-3400SD pump, an autosampler WPS-3000 TSL, a column compartment TCC-3000SD and a diode array detector DAD3000 (monitoring from 254-720 nm). Mixtures of acetonitrile (ACN) and aqueous 20 mM NH_4OAc were used as eluents in a linear gradient system (5 min at 25% ACN, 5-30 min to 95% ACN, 30-35 min at 95% and 35-45 min at 25% ACN)

Chemical names of compounds were generated by ChemDraw Professional 16.0.

(*E*)-1'-(3-(4-chlorophenyl)allyl)-5-fluorospiro[indoline-3,4'-piperidine] (**1**): The already published derivative **1** was synthesized in quantities of 1.6 g according to the procedure as described.[28] R_f = 0.54 (ethyl acetate/*n*-hexane/NH_3 3:1:0.1); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.75 (d; *J* = 11.1 Hz; 2H), 1.92 (td; *J* = 12.6 Hz; *J* = 4.0 Hz; 2H), 2.11 (td; *J* = 11.9 Hz; *J* = 2.6 Hz; 2H), 2.94 (d; *J* = 11.9 Hz; 2H), 3.17 (dd; *J* = 6.7 Hz; *J* = 1.3 Hz; 2H), 3.44 (s; 2H), 3.56 (bs; -NH), 6.28 (dt; *J* = 15.9 Hz; *J* = 6.7 Hz; 1H), 6.46 (s; 1H), 6.52 (dd; *J* = 4.4 Hz; *J* = 4.2 Hz; 1H), 6.72 (ddd; *J* = 9.1 Hz; *J* = 8.4 Hz; *J* = 2.6 Hz; 1H), 6.79 (dd; *J* = 8.6 Hz; *J* = 2.6 Hz; 1H), 7.24-7.32 (m; 4H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz): δ= 35.9, 44.7 (*J* = 1.8 Hz), 50.9, 61.3, 77.29, 110.0 (*J* = 2.1 Hz), 110.1 (*J* = 30.3 Hz), 113.7 (*J* = 23.5 Hz), 127.4, 127.5, 128.7, 131.8, 133.1, 135.4, -, 138.8 (*J* = 6.9 Hz), 146.4, 157.1 (*J* = 235.5 Hz) ppm; ^{19}F NMR ($CDCl_3$, 282 MHz): δ= -125.73 ppm; MS (ESI-(+)): *m/z* calcd. for $C_{21}H_{23}ClFN_2$ $[M+H]^+$ 357.1; found 357.2.

***(E)*-(1'-(3-(4-chlorophenyl)allyl)-5-fluorospiro[indoline-3,4'-piperidin]-1-yl)(2-chloropyridin-4-yl)methanone** (**2, SYN876**): The synthesis of the already published derivative **2** was slightly modified by using 2-chloroisonicotinic acid instead of the corresponding acid chloride as described.[28] 2-Chloroisonicotinic acid (0.043 g; 0.308 mmol; 1.9 eq.), 0.057 g (0.159 mmol; 1.0 eq.) **1**, 0.161 g (0.364 mmol; 2.3 eq.) BOP and 0.12 mL (0.085 g; 0.840 mmol; 5.3 eq.) triethylamine (TEA) were dissolved in 8 mL dichloromethane under argon atmosphere. The solution was stirred for 24 h. Then 50 mL water were added and the aqueous layer was extracted with $CHCl_3$ (4 x 25 mL). The combined organic

layers were dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by column chromatography (SiO_2; ethyl acetate/*n*-hexane/NH_3 1:1:0.1) to afford the titled compound **2** (0.059 g; 0.123 mmol; 77%). R_f = 0.48 (ethyl acetate/*n*-hexane/NH_3 1:1:0.1); ^{1}H NMR (DMSO-d6, 400 MHz): δ= 1.66 (m; 2H), 1.87 (m, 4H), 2.80 (s; 2H), 3.04 (m, 2H), 3.87 (s; 2H), 6.29 (dt; *J* = 6.2 Hz; *J* = 13.2 Hz; 1H), 6.49 (d; *J* = 15.9 Hz; 1H), 7.09 (t; *J* = 7.9 Hz; 1H), 7.26 (m; 1H), 7.35 (d; *J* = 8.4 Hz; 2H), 7.44 (d; *J* = 8.3 Hz; 2H), 7.66 (d; *J* = 4.4 Hz; 1H), 7.78 (s; 1H), 8.12 (dd; *J* = 4.7 Hz; *J* = 8.3 Hz; 1H), 8.59 (d; *J* = 5.0 Hz; 1H) ppm; ^{13}C NMR (DMSO-d6, 100 MHz): δ= 36.3, 43.5, 50.4, 59.6, 60.6, 110.9 (*J* = 24.4 Hz), 114.5 (*J* = 23.1 Hz), 118.3 (*J* = 7.6 Hz), 121.0, 122.3, 128.3, 128.8, 129.0, 131.1, 132.2, 136.1, 138.0, 143.5 (*J* = 7.4 Hz), 147.7, 151.2 (*J* = 5.9 Hz), 159.9 (*J* = 240.5 Hz), 164.7 ppm; ^{19}F NMR (DMSO-d6, 376 MHz): δ= -117,0 ppm; HRMS (ESI-(+)): *m/z* calcd. for $C_{27}H_{25}Cl_2FN_3O$ $[M+H]^+$ 496.1246; found 496.1351 $[M+H]^+$.

(*E*)-(1'-(3-(4-chlorophenyl)allyl)-5-fluorospiro[indoline-3,4'-piperidin]-1-yl)(2-fluoropyridin-4-yl)methanone (**3**): The synthesis of the already published derivative **3** was slightly modified by using fluoroisonicotinic acid instead of the corresponding acid chloride as described.[28] 2-Fluoroisonicotinic acid (0.043 g; 0.308 mmol; 1.9 eq.), 0.057 g (0.159 mmol; 1.0 eq.) **1**, 0.161 g (0.364 mmol; 2.3 eq.) BOP and 0.12 mL (0.085 g; 0.840 mmol; 5.3 eq.) triethylamine were dissolved in 8 mL dichloromethane under argon atmosphere. The solution was stirred for 24 h. Then 50 mL water were added and the aqueous layer was extracted with $CHCl_3$ (4 x 25 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by column chromatography (SiO_2; ethyl acetate/*n*-hexane/NH_3 1:1:0.1) to afford the titled compound **3** (0.059 g; 0.123 mmol; 77%). R_f = 0.42 (ethyl acetate/*n*-hexane/NH_3 1:1:0,1); ^{1}H NMR ($CDCl_3$, 400 MHz): δ= 1.72 (m; 2H), 1.99 (m; 4H), 3.01 (m; 2H), 3.16 (s; 2H), 3.80 (s; 2H), 6.24 (m; 1H), 6.48 (d; *J* = 15.6 Hz; 1H), 6.95 (dd; *J* = 2.4 Hz; *J* = 8.1 Hz; 1H), 7.00 (m; 1H); 7.09 (s; 1H), 7.29-7.32 (m; 5H), 8.20 (bs; 1H), 8.41 (d; *J* = 4.6 Hz; 1H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 36.4, 43.5, 50.6, 60.5, 61.1, 107.6 (*J* = 38.6 Hz), 110.1 (*J* = 24.1 Hz), 114.8 (*J* = 24.5 Hz), 118.9 (*J* = 14 Hz), 119.0, 127.0, 127.5, 128.8, 132.1, 133.3, 135.2, 137.0, 142.0, 149.0 (*J* = 14.9 Hz), 149.3, 160.4 (*J* = 243.7 Hz), 163.9 (*J* = 241.3 Hz), 164.5 ppm; ^{19}F NMR ($CDCl_3$, 376 MHz): δ = -65.1, -117.6 ppm; HRMS (ESI-(+)): *m/z* calcd. for $C_{27}H_{25}ClF_2N_3O$ $[M+H]^+$ 480.1649; found 480.1647 $[M+H]^+$.

(*E*)-(6-bromopyridin-3-yl)(1'-(3-(4-chlorophenyl)allyl)-5-fluorospiro[indoline-3,4'-piperidin]-1-yl)methanone (**4**): 6-Bromopyridine-3-carboxylic acid (0.13 g, 0.672 mmol, 1.2 eq.), 0.20 g (0.560 mmol; 1.0 eq.) **1**, 0.370 g (0.840 mmol; 1.5 eq.) BOP and 0.27 mL (0.20 g; 1.960 mmol; 3.5 eq.) triethylamine were dissolved in 15 mL dichloromethane under argon atmosphere. The solution was stirred for 24 h. Additional 0.056 g (0.280 mmol; 0.5 eq.) 6-bromopyridine-3-carboxylic acid, 0.124 g

(0.280 mmol; 0.5 eq.) BOP and 76.7 µl (0.056 g; 0.560 mmol; 1.0 eq.) triethylamine were added and stirred for further 48 h. After addition of 50 mL water, the aqueous layer was extracted with $CHCl_3$ (4 x 25 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by column chromatography (SiO_2; ethyl acetate/*n*-hexane/NH_3 1:1:0.1) to afford the titled compound **4** (0.102 g; 0.188 mmol; 34%). R_f = 0.30 (ethyl acetate/*n*-hexane/NH_3 1:1:0.1); ^{1}H NMR (DMSO-d6, 300 MHz): δ = 1.64 (m; 2 H), 1.87 (m; 4 H), 2.8 (m; 2 H), 3.05 (m; 2 H), 3.93 (s; 2 H), 6.28 (dt; *J* = 15.7 Hz; *J* = 6.4 Hz; 1 H), 6.48 (d; *J* = 15.8 Hz; 1 H), 7.03 (m; 1 H), 7.23 (dd; *J* = 8.7 Hz; *J* = 2.7 Hz; 1 H), 7.33 (d; *J* = 8.6 Hz; 2 H), 7.42 (d; *J* = 8.5 Hz; 2 H), 7.79 (d; *J* = 8.1 Hz; 1 H), 8.02 (m; 3 H), 8.65 (m; 1 H) ppm; ^{13}C NMR (DMSO-d6, 75 MHz): δ= 36.2, 43.3, 50.4, 59.9, 60.6, 110.9 (*J* = 22.9 Hz), 114.4 (*J* = 23.2 Hz), 118.2 (*J* = 8.4 Hz), 128.3, 128.5, 128.7, 129.0, 131.2, 132.2, 132.6, 136.0, 138.2, 138.7, 143.2, 143.5, 149.3, 159.7 (*J* = 240.5 Hz), 165.2 ppm; ^{19}F NMR (DMSO-d6, 282 MHz): δ= -117.3 ppm; HRMS (ESI-(+)): m/z calcd. for $C_{27}H_{25}BrClFN_3O$ $[M+H]^+$ 540.0854; found 540.0846.

(*E*)- (1'-(3-(4-chlorophenyl)allyl)-5-fluorospiro[indoline-3,4'-piperidin]-1-yl)(6-fluoropyridin-3-yl)methanone (**5**): 6-Fluoropyridine-3-carboxylic acid (0.095 g, 0.672 mmol, 1.2 eq.), 0.20 g (0.560 mmol; 1.0 eq.) **1**, 0.370 g (0.840 mmol; 1.5 eq.) BOP and 0.27 mL (0.20 g; 1.960 mmol; 3.5 eq.) triethylamine were dissolved in 15 mL dichloromethane under argon atmosphere. The solution was stirred for 48 h. Then additional 0.039 g (0.280 mmol; 0.5 eq.) 6-fluoropyridine-3-carboxylic acid, 0.124 g (0.280 mmol; 0.5 eq.) BOP and 76.7 µL (0.056 g; 0.560 mmol; 1.0 eq.) TEA were added and stirred for further 48 h. After addition of 50 mL water, the aqueous layer was extracted with $CHCl_3$ (4 x 25 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by column chromatography (SiO_2; ethyl acetate/*n*-hexane/NH_3 1:1:0.1) to afford the titled compound **5** (0.118 g; 0.246 mmol; 44%). R_f = 0.16 (ethyl acetate/*n*-hexane/NH_3 1:1:0.1); ^{1}H NMR (DMSO-d6, 400 MHz): δ = 1.64 (m; 2H), 1.85 (m; 4 H), 2.80 (m; 2H), 3.04 (d; *J* = 6.4 Hz; 2H), 3.94 (s; 2H), 6.27 (dt; *J* = 15.9 Hz; *J* = 6.4 Hz; 1H), 6.47 (d; *J* = 15.9 Hz; 1H), 7.03 (bs; 1H), 7.22 (dd; J = 8.7 Hz; J = 2.7 Hz; 1H), 7.32 (m; 3H), 7.41 (m; 2H), 8.09 (bs; 1H), 8.27 (m, 1H), 8.54 (bs; 1H) ppm; ^{13}C NMR (DMSO-d6, 100 MHz): δ= 36.2, 43.3, 50.4, 60.0, 60.6, 110.2 (*J* = 37.5 Hz), 110.8 (*J* = 23.8 Hz), 114.3 (*J* = 23.1 Hz), 115.7 (*J* = 22.5 Hz), 118.2, 128.3, 128.7, 129.0, 131.1, 131.6, 132.2, 136.0, 138.2, 141.8 (*J* = 8.7 Hz), 143.5 (*J* = 7.8 Hz), 147.2 (*J* = 15.8 Hz), 159.6 (*J* = 240.6 Hz), 163.8 (*J* = 239.0 Hz), 165.3 ppm; ^{19}F NMR (DMSO-d6, 282 MHz): δ = -65.9, -117.4 ppm; HRMS (ESI-(+)): m/z calcd. for $C_{27}H_{25}ClF_2N_3O$ $[M+H]^+$ 480.1654; found 480.1647.

(*E*)-(1'-(3-(4-chlorophenyl)allyl)-5-fluorospiro[indoline-3,4'-piperidin]-1-yl)(4-fluorophenyl)methanone (**6**): In a round bottom flask 0.072 g (0.202 mmol; 1.0 eq.) of **1**, 0.034 g

(0.242 mmol; 1.2 eq.) of 4-fluorobenzoic acid, 0.134 g (0.303 mmol; 1.5 eq.) BOP and 0.10 mL (0.071 g; 0.707 mmol; 3.5 eq.) of triethylamine were dissolved in 8 mL dichloromethane under argon atmosphere. The solution was stirred for 1 day at room temperature. Then 50 mL of water were added and the aqueous layer was extracted with chloroform (4 × 25 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by column chromatography (SiO_2; ethyl acetate/*n*-hexane/NH_3 1:1:0.1) to afford the titled compound **6** (0.029 g; 0.060 mmol; 30%). R_f = 0.56 (ethyl acetate/*n*-hexane/NH_3 1:1:0,1); ^{1}H NMR (DMSO-d6, 300 MHz): δ = 1.64 (m; 2H), 1.87 (m; 4H), 2.82 (m; 2H), 3.05 (m; 2H), 3.89 (s; 2H), 6.29 (dt; *J* = 6.4 Hz; *J* = 15.7 Hz; 1H), 6.49 (d; *J* = 15.9 Hz; 1H), 7.02 (br s; 1H), 7.22 (dd; *J* = 2.6 Hz; *J* = 8.6 Hz; 1H), 7.33 (m; 4H), 7.43 (m; 2H), 7.67 (m; 2H), 8.02 (br s; 1H) ppm; ^{13}C NMR (DMSO-d6, 75 MHz): δ= 36.1, 43.1, 50.5, 60.2, 60.7, 110.8 (*J* = 24.1 Hz); 114.3 (*J* = 23.1 Hz), 116.0 (*J* = 21.7 Hz), 118.1, 128.3, 128.7, 129.0, 130.2 (*J* = 8.6 Hz), 131.2, 132.2, 133.5, 136.0, 138.4, 143.4 (*J* = 7.1 Hz), 159.5 (*J* = 240.3 Hz), 163.4 (*J* = 247.8 Hz), 167.4 ppm; ^{19}F NMR (DMSO-d6, 282 MHz): δ= -110.0, -117.9 ppm; HRMS (ESI-(+)): *m/z* calcd. for $C_{28}H_{26}ClF_2N_2O$ $[M+H]^+$ 479.1701; found 479.1693.

2-Fluoroethyl (*E*)-1'-(3-(4-chlorophenyl)allyl)-5-fluorospiro[indoline-3,4'-piperidine]-1-carboxylate (**7**): In a round bottom flask 0.044 g (0.123 mmol; 1.0 eq.) of **1**, 0.026 g (0.185 mmol; 1.5 eq.) of 2-fluoroethyl methanesulfonate and 0.061 g (0.369 mmol; 3.0 eq.) potassium carbonate were dissolved in 2 mL DMF under argon atmosphere and stirred at 90 °C for 19 h. Then additional 2-fluoroethyl methanesulfonate (8.7 mg, 0.061 mmol, 0.5 eq.) was added and the mixture heated for further 10 h. After addition of 50 mL of water, the aqueous layer was extracted with chloroform (4 × 25 mL) and the combined organic layers were dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by column chromatography (SiO_2; ethyl acetate/*n*-hexane/NH_3 1:2:0.1) to afford the titled compound **7** (0.029 g; 0.065 mmol; 54%). R_f = 0.27 (ethyl acetate/*n*-hexane/NH_3 1:2:0.1); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.70 (d; *J* = 12.7 Hz; 2H), 1.95 (td; *J* = 3.3 Hz; *J* = 12.9 Hz; 2H), 2.12 (t; *J* = 12.0 Hz; 2H), 3.00 (d; *J* = 11.6 Hz; 2H), 3.20 (d; *J* = 6.7 Hz; 2H), 3.91 (s; 2H), 4.47 (d; *J* = 28.3 Hz; 2H), 4.70 (d; *J* = 47.6 Hz; 2H), 6.28 (dt; *J* = 6.67 Hz; *J* = 14.3 Hz; 1H), 6.50 (d; *J* = 15.9 Hz; 1H), 6.86 (s; 1H), 6.88 (s; H), 7.27-7.33 (m; 4H), 7.79 (s; 1H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 37.1, 42.4, 50.5, 57.7, 61.3, 64.4 (*J* = 20.8 Hz), 81.7 (*J* = 170.5 Hz), 110.1 (*J* = 23.1 Hz), 114.5 (*J* = 23.1 Hz), 115.6, 127.1, 127.5, 128.7, 132.0, 133.2; 135.3; 137.4; 140.6; 152.4; 159.2 (*J* = 241.3 Hz) ppm; ^{19}F NMR ($CDCl_3$, 282 MHz): δ= 5.3, -119.9 ppm; HRMS (ESI-(+)): m/z calcd. for $C_{24}H_{26}ClF_2N_2O_2$ $[M+H]^+$ 447.1645; found 447.1656.

5.2. *In vitro* binding experiments

5.2.1 Preparation of homogenates of rVAChT-PC12 cells

rVAChT-PC12 cells (by courtesy of Prof. Ali Roghani, Texas Tech University, Lubbock, USA) were grown in DMEM (high glucose, 5% cosmic calf serum, 10% equine serum, 100 U/mL penicillin-streptomycin, 0.125 g/L G-418) to confluence (37 °C, 5% CO_2). Cells were harvested by scraping into the medium and pelleted by centrifugation (800 rpm, 3 min). The cell pellet was resuspended in chilled 50 mM TRIS-HCl, pH 7.4 and triturated to effect hypotonic lysis. The suspension was centrifuged at 15.000 rpm at 4 °C for 15 min. The obtained crude membrane fraction was maintained at -25 °C until assay. For the binding experiments aliquots were thawed and resuspended in a desired volume of assay buffer.

5.2.2 Preparation of rat brain and liver tissue homogenates

The studies involving experiments with animal tissues were performed in accordance with the national law on the care and use of laboratory animals and approved by the local Ethics Committee (Landesdirektion Sachsen, T 48/14).

Female Sprague Dawley rats (150–200 g) were anaesthetized and decapitated. Their brains and livers were rapidly removed from the skull. Brain tissue without cerebellum as well as liver samples were homogenized in 10 volumes (w/v) of chilled 50 mM TRIS-HCl, pH 7.4, using a Teflon-glass homogenizer (10 strokes, 1500 rpm). The respective homogenates were centrifuged (14.000 rpm, 4°C, 15 min), the resulting supernatants were decanted and the pellets resuspended with the same buffer. The homogenization-centrifugation was performed three times, and the finally obtained washed pellets were aliquoted in the same buffer and stored at -25 °C until assay.

5.2.3 Binding experiments

For VAChT binding assays, the crude membrane fraction of VAChT-PC12 cells was incubated with (-)-[^{3}H]vesamicol ([^{3}H]2-(4-phenylpiperidinyl)cyclohexanol, molar activity 1258 GBq/mmol, PerkinElmer Life Sciences, Boston, MA, USA; 1.5 nM) and up to seven concentrations of the test compound (0.01 nM – 10 µM) in a volume of 1 mL (50 mM TRIS-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$) in duplicate for 120 min at room temperature under agitation (200 min^{-1}). Nonspecific binding of the radioligand was determined by co-incubation with 10 µM (±)-vesamicol.

For comparison of the bindings sites of compound **2** and vesamicol at VAChT, competition assays were performed as described above by using (±)-vesamicol as test compound alone or in the presence of 100 nM or 1000 nM compound **2** in the same assay, i.e., with absolute identical test conditions.

For σ_1 and σ_2 receptor binding assays, the crude membrane fractions of rat brain and liver were incubated with (+)-[^{3}H]pentazocine (specific activity: 1070 GBq/mmol, PerkinElmer Life Sciences,

Boston, USA) and [^{3}H]DTG (specific activity: 1110 GBq/mmol, PerkinElmer Life Sciences, Boston, USA) in the presence of 1 µM of the σ_1 receptor masking dextrallorphan (by courtesy of Roche AG, Basel, Switzerland), respectively, in a volume of 1 mL (50 mM TRIS-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$) in duplicate for 120 min at room temperature under agitation (200 min^{-1}). Nonspecific binding of both radioligands was determined in the presence of 10 µM haloperidol.

The incubations were terminated by rapid filtration through Whatman GF/B glass-fiber filters presoaked in 0.3% polyethylenimine for 60 min at room temperature using a rapid vacuum filtration system (cell harvester Brandel Gaithersburg, MD, USA), and the filters were washed three times with 4 mL chilled 50 mM TRIS-HCl, pH 7.4. Filterbound radioactivity was measured by liquid scintillation counting (Tri-Carb2900TR, PerkinElmer Life Sciences Boston, MA, USA).

IC_{50} values were calculated from competitive binding curves by nonlinear curve fitting (GraphPad Prism, version 3; GraphPad Software, Inc., San Diego, USA). The apparent inhibition constant (K_i) was derived from IC_{50} values according to the CHENG–PRUSOFF equation: $K_i = IC_{50}/(1+C/K_D)$ where C is the concentration of the radioligand and K_D is the dissociation constant of the radioligand (in-house determined values: K_D (-)-[^{3}H]vesamicol = 25.6 nM to VAChT on rVAChT-PC12 cells, K_D (+)-[^{3}H]pentazocine = 6.9 nM to σ_1 receptors on rat cortical membranes, K_D [^{3}H]DTG = 29 nM for σ_2 receptors on rat liver membranes with masking of σ_1 receptors using dextrallorphan).

Linear transformation according to Hanes-Woolf was performed using the protocols implemented in GraphPad Prism (Version 3; GraphPad Software, Inc., San Diego, USA).

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Supplementary material

Supplementary material associated with this article can be found in the online version at:

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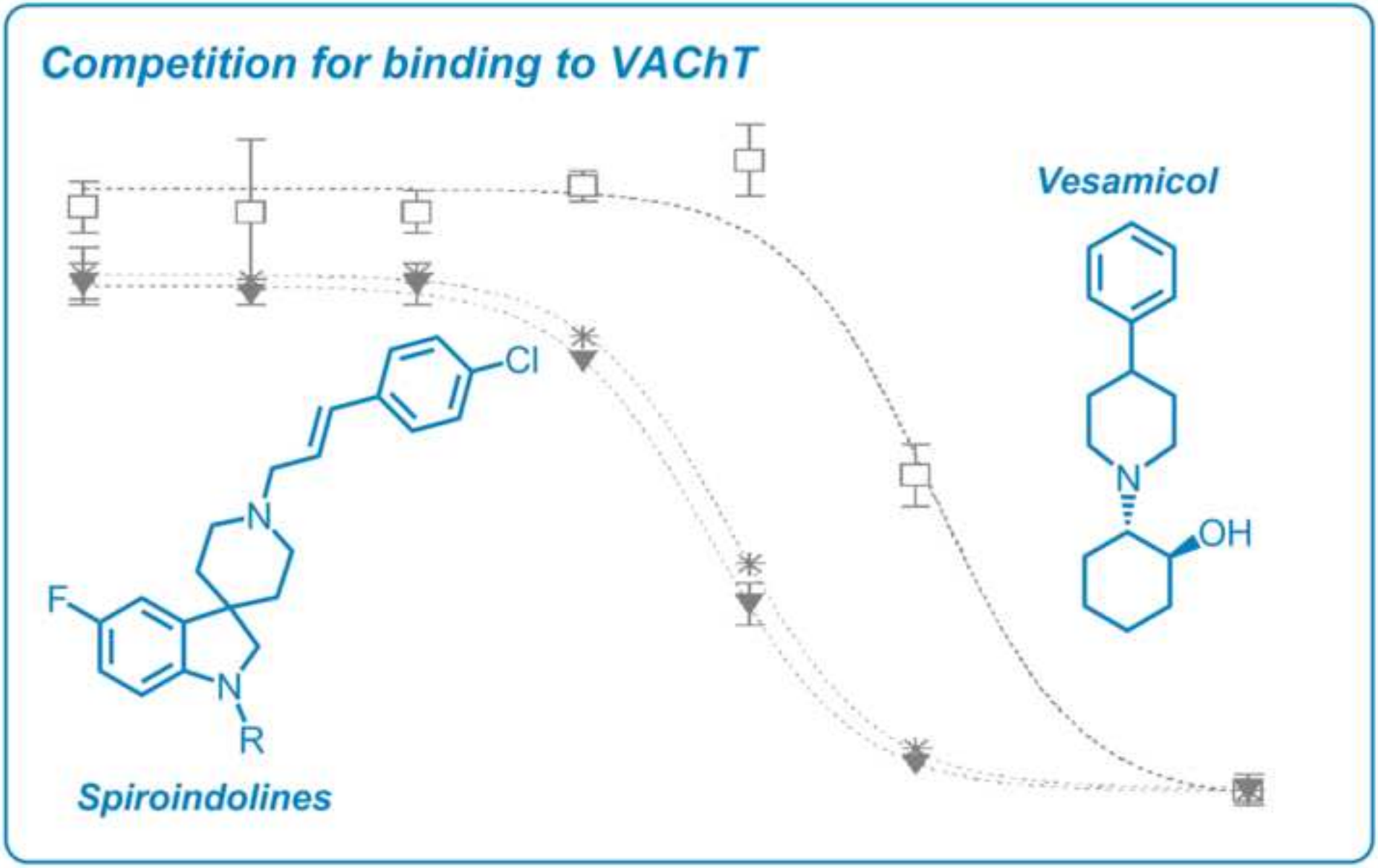
Figure captions:

Figure 1. Known VAChT ligands vesamicol, benzovesamicol and spiroindoline [^{3}H]SYN876 as potential lead compounds in the development of VAChT PET radiotracers

Figure 2. Representation of binding data as non-linearized inhibition curves (one out of three experiments; n=3 per data point).

Figure 3. Representation of binding data after Hanes-Woolf linearization.

Scheme 1. Reagents and conditions: a) BOP, TEA, 2-chloroisonicotinic acid for **2**, 2-fluoroisonicotinic acid for **3**, 6-bromonicotinic acid for **4**, 6-fluoronicotinic acid for **5** and 4-fluorobenzoic acid for **6**, CH_2Cl_2, rt, 24 h; b) K_2CO_3, DMF, 2-fluoroethyl methanesulfonate, 90 °C, 19 h.


Competition for binding to VAChT
Vesamicol
OH
N
Cl
F
N
N
R
Spiroindolines