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In vitro binding profile and radiosynthesis of a novel ^{18}F-labeled azaspirovesamicol analog as potential ligand for imaging of the vesicular acetylcholine transporter

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Radiolabeled vesamicol analogs are promising candidates as ligands for the vesicular acetylcholine transporter (VAChT) to enable *in vivo* imaging of early cholinergic degenerations in brain. The 4-fluorobenzoyl-substituted azaspirovesamicol derivative FBASV is one out of six novel vesamicol analogs and demonstrated most appropriate *in vitro* binding data. ^{18}F-radiolabeling was performed by microwave-assisted nucleophilic aromatic substitution of the corresponding nitro precursor and two methods were developed for the purification of [^{18}F]FBASV. Utilizing method A, the remaining nitro precursor was reduced to its corresponding amine, which was separated via semi-preparative HPLC on a conventional RP column. In method B a phenyl column was used for the direct separation of [^{18}F]FBASV and its nitro precursor, resulting in a change of the elution order and better separation parameters. Thus, [^{18}F]FBASV was synthesized with a RCY of 16–18%, a specific activity >300 GBq/μmol, and a radiochemical purity of >99.5% suitable for future *in vivo* studies.

Keywords: azaspirovesamicol; [^{18}F]FBASV; VAChT; PET; ^{18}F-labeling; vesamicol; nitro precursor

Introduction

One of the most significant neuropathological findings in Alzheimer's disease is the degeneration of cholinergic neurons in the brain associated with a progressive impairment of cognitive function. Exclusively located in presynaptic nerve terminals, the vesicular acetylcholine transporter (VAChT) was intensely studied as valuable target for *in vivo* imaging of neurodegenerative processes using SPECT and PET,[1,2] with PET being superior regarding detection efficiency, spatial resolution and quantification. All VAChT-radioligands synthesized so far are based on the structure of vesamicol [2-(4-phenylpiperidin-1-yl) cyclohexanol], a drug that binds with low-nanomolar affinity to an allosteric site of the transporter protein.[3–6] However, vesamicol itself is not suitable as radioligand due to its insufficient selectivity expressed by the additional binding to the σ_1 and σ_2 receptors,[7] which are also present in cholinergic brain regions. As the transporter protein only tolerates vesamicol-like structures, the development of VAChT radiotracers has focused on chemical modifications of vesamicol as chemical lead and resulted in three main groups of derivatives: (i) benzovesamicols,[8–10] (ii) trozamicols,[11–13] and (iii) morpholino vesamicols,[14–16] the latter class is currently processed in our group. In particular, the benzovesamicol and trozamicol derivatives are characterized by sub-nanomolar affinities towards VAChT; however, some of them bind with nanomolar affinities to the sigma receptors.[17] Because of this moderate selectivity and due to undesired side effects none of these ligands has been really accepted for clinical application. By contrast, the morpholino vesamicols show nanomolar affinities towards VAChT and almost no affinity to sigma receptors resulting in promising first *in vivo* data.[15] Recently we developed the azaspirovesamicols,[18,19] a second novel class of structurally modified vesamicol analogs with markedly low sigma receptor affinity. In particular the 4-fluorobenzoyl-substituted derivative FBASV ([4-fluorophenyl][9-hydroxy-8-(4-phenylpiperidin-1-yl)-2-azaspiro[5.5]undecan-2-yl]methanone] was characterized by its appropriate binding affinity towards VAChT and high selectivity *in vitro* to develop them as ^{18}F-labeled radiotracer. In this report, we describe (i) the *in vitro* target affinity and selectivity of six azaspirovesamicol derivatives and (ii) two alternative approaches for the radiosynthesis of **[^{18}F]FBASV**, the most promising candidate of this novel class of VAChT ligands.

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Experimental

Chemistry

Mass spectra were recorded on a Mariner Biospectrometer Workstation (Applied Biosystems) using ElectroSpray Ionization (ESI). Analytical thin-layer chromatography (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. Analytical chromatographic separations of the isomers **1a**–**1d** and **4a**–**4b** were performed on a JASCO LC-2000 system, incorporating a PU-2080*Plus* pump, AS-2055*Plus* auto injector (100 μL sample loop), and a UV-2075*Plus* UV detector (monitoring at 254 nm) by using a 250 × 4.6 mm Nucleodur Sphinx RP – 5 μm column (Machery-Nagel, Germany). For the semi-preparative separation of the isomer **4a** a Merck-Hitachi system D-7000 with an L-7100 pump, a Rheodyne injection valve with a 500 μL sample loop, a L-7400 UV detector, and a 250 × 10.0 mm Nucleodur Sphinx RP – 5 μm column were used.

*(±)-9-Hydroxy-(8-(4-phenylpiperidin-1-yl)-2-azaspiro[5.5]undecan-2-yl)(4-nitrophenyl)methanone (**4a**)*

Ten millilitre 6M aqueous HCl was added to a solution of 600 mg (1.3 mmol) of the isomeric mixture of **1a**–**1d** in 6 mL ethanol. The mixture was refluxed for 3 days. After cooling at room temperature, the solution was washed with methyl-*tert*-butyl-ether (MTBE) (3 × 1 mL) and adjusted to pH 11 with 6M aqueous NaOH. Thus, organic basic components could be extracted with CH_2Cl_2 (5 × 5 mL) and the organic phase was dried with Na_2CO_3. Removal of the solvent provided a yellow oily residue which was purified via column chromatography (silica gel, $CHCl_3$/MeOH/NH_3 10:2:0.2) to get 328 mg (77%) of the corresponding secondary amines as nearly colorless oil. MS (ESI) for $C_{21}H_{32}N_2O$: *m/z* 329.26 $[M+H]^+$.

In total, 289 mg (0.88 mmol) of this product were dissolved in 8 mL CH_2Cl_2 and flushed with argon. To this solution, 148 mg (1.76 mmol) $NaHCO_3$ in 3 mL water and 163 mg (0.88 mmol) NO_2-benzoyl chloride in 5 mL CH_2Cl_2 were added. After stirring at room temperature for 3 h, the organic phase was washed with water, dried with Na_2CO_3 and the solvent was removed under vacuum. The residual yellowish oil (404 mg, 96%) was used for semi-preparative HPLC to separate the isomer **4a**. Analytical HPLC of the crude product (Nucleodur Sphinx 250 × 4.6 mm; 95% MeCN/20 mM NH_4OAc aq.; 0.5 mL/min) provided following retention times: t_R = 14.5–15.4 (impurities), t_R(**4a**) = 21.7, t_R(**4b**) = 27.1; t_R(**4c**) = 27.9; t_R(**4d**) = 30.9 min. For the semi-preparative separation a Nucleodur Sphinx 250 × 10 mm column was used with an eluent composition of 95% MeCN/20 mM NH_4OAc aq. When 3.5 mg of the crude product dissolved in 500 μL MeCN were injected at a flow rate of 1.2 mL/min, the precursor **4a** eluted with a t_R = 27 min and could be collected without impurities of the other isomers. Conveniently, this sample was injected ten times every 30 min, without stopping the run. The collected fractions were concentrated to a volume of about 10 mL, adjusted to pH 11 with 6 M aqueous NaOH and extracted with CH_2Cl_2 (3 × 5 mL). The organic phases were washed with water and dried with Na_2CO_3. Removal of the solvent gave 10.5 mg (33% of the crude product) of **4a** as a white solid, whose purity was controlled by HPLC. MS (ESI) for $C_{28}H_{35}N_3O_4$: *m/z* 478.26 $[M+H]^+$.

In vitro binding experiments

The affinities of **1a**–**3b** to VAChT binding sites, expressed by their K_i values, were determined by competitive binding assays using rat pheochromocytoma PC12 cells stably transfected with cDNA of rat VAChT (VAChT-PC12) and (−)-[^{3}H]vesamicol as radioligand. The concentration necessary to inhibit 50% of the binding of (−)-[^{3}H]vesamicol (IC_{50} value) was determined for each compound.

To evaluate the affinities of **1a**–**3b** to sigma binding sites, VAChT-PC12 cells were used, because these cells also express σ_1 and σ_2 receptors[20,21].

Assays were performed in 50 mM TRIS/HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$.

VAChT-PC12 cells were grown in Dulbecco's modified Eagle Medium with 4.5 g/L D-glucose and supplemented with 5% cosmic calf serum, 10% equine serum, and 100 U/mL penicillin–streptomycin. Cells were harvested using a cell scraper, washed twice with ice-cold PBS buffer, disrupted by 15 strokes in a Dounce homogenizer, rotated at 5000 g and homogenized in assay buffer. Cell counting was performed in a Neubauer chamber after cell washing and staining with methylene blue.

For the standard assay 1×10^6 VAChT-PC12 cells were incubated with 3 pmol (−)-[^{3}H]vesamicol (specific activity: 1258 GBq/mmol, PerkinElmer Life Sciences, Boston, USA) and the compounds to be tested (concentrations between 10^{-11} and 10^{-5} mol/L) in a volume of 1 mL in duplicates for 2 h at 25°C under agitation (200 min^{-1}). Nonspecific binding was determined in presence of 10 μM (−)-vesamicol.

The determination of binding of **1a**–**3b** to sigma receptors was performed in the same way with 3 pmol/mL [^{3}H]DTG (specific activity: 1110 GBq/mmol, PerkinElmer Life Sciences, Boston, USA) as radioligand. Nonspecific binding was determined in the presence of 10 μM DTG.

After incubation, bound and free radioligand were separated by rapid vacuum filtration through Whatman GF/B glass-fiber filters presoaked for 3 h in 0.5% polyethyleneimine using a Brandel cell harvester (Gaithersburg, MD, USA). The filters were washed three times with 4 mL ice-cold TRIS buffer (0.05 mol/L, pH 7.4). The dried filters were slightly shaken in 10 mL Rotiscint eco plus cocktail (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for about 30 min, maintained in the dark for at least 8 hours and measured in a Tri-Carb2900TR Liquid Scintillation Counter (PerkinElmer, Boston, MA, USA) with 70% counting efficiency to obtain dpm values as measures of radioactivity.

IC_{50} values of **1a**–**3b** were calculated from competitive binding curves by a nonlinear curve fitting, using Graphpad Prism, version 3 (GraphPad Software, Inc., San Diego, USA). Specific binding of the radioligand was defined as total binding minus non-specific binding. 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. The K_D value of (−)-[^{3}H]vesamicol for rat VAChT-PC12 cells was recently determined as 3.74 ± 0.2 nM.[22] The K_D value of [^{3}H]DTG for sigma receptors expressed in VAChT-PC12 cells is 39.8 nM (own unpublished data).

Radiochemistry

Aqueous [^{18}F]fluoride was produced with a PETtrace cyclotron (GE Healthcare). Microwave-assisted radiosyntheses were carried

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out in a standard 10 mL vial using a research microwave reactor equipment Discover® (CEM, Germany). For Solid Phase Extraction, Sep-Pak® C18 Plus cartridges (Waters, USA) were used in RP mode. TLC was performed on silica gel pre-coated plates (Polygram® SIL G/UV_{254}) with ethyl acetate (EE)/n-hexane/NH_3 8.5:1.5:0.2 (v/v/v) or EE/MeOH 3:1 and the spots were visualized using UV light at 254 nm. Both eluents provided similar results: R_f (**4a**) = 0.54, R_f (**[^{18}F]FBASV**) = 0.63 using EE/n-hexane/NH_3 and R_f (**4a**) = 0.51, R_f (**[^{18}F]FBASV**) = 0.45 using EE/MeOH 3:1. Radioluminescence thin layer chromatograms (radio-TLC) were recorded using a BAS-1800 II system Bioimaging Analyzer (Fuji Film, Japan) and images were evaluated with AIDA 2.31 software (raytest, Germany).

The crude labeling product was separated on a semi-preparative radio-HPLC system consisting of a S1021 pump (SYKAM Chromatographie, Germany), a UV detector (WellChrom K-2001; KNAUER, Germany), and a NaI(Tl)-counter. For data acquisition a Nina Chromatografix system (Nuclear Interface, Germany) was used. Analytical radio-HPLC was performed on an advanced Agilent HP 1100 HPLC system including a binary pump, an autosampler, a variable wavelength UV detector (monitoring at 220, 228, 254, and 344 nm), and a NaI(Tl)-counter.

*Radiosyntheses of **[^{18}F]FBASV***

To get the [^{18}F]KF-K2.2.2-carbonate complex, aqueous [^{18}F]fluoride was diluted with an aqueous solution of K_2CO_3 (1.78 mg, 12.9 μmol) and azeotropically dried in MeCN in the presence of Kryptofix 2.2.2 (11.2 mg, 29.7 μmol). The resulting reactive anhydrous [^{18}F]KF-K2.2.2-carbonate complex was dissolved in 0.5 mL extra dry DMF and transferred to a standard glass microwave reaction vial (10 mL) containing 3.0 mg of the nitro precursor **4a** dissolved in 1 mL extra dry DMF. The mixture was heated to 154–156°C and irradiated at 75 W in a single mode microwave cavity for 25 min. Afterwards, the solution was cooled in an ice-bath, diluted with 50 mL of water and passed through a Sep-Pak C18 cartridge. The crude product was eluted with 2 mL MeOH (method A) or MeCN (method B).

Method A: After drying of the yellowish MeOH eluate with Na_2SO_4, a catalytic amount of 5% Pd on $BaSO_4$ ($\sim$4 mg) and 15–20 mg ammonium formate were added. The reduction of residual nitro precursor **4a** was achieved at 65°C for 20 min. The precipitated palladium was removed completely by centrifugation, and the supernatant was loaded on a Sep-Pak C18 cartridge. The product was eluted with 2 mL MeCN, diluted with 2 mL water and applied to semi-preparative radio-HPLC. Using a Multospher 120 RP AQ column (150 × 10 mm ID) and 52% MeCN/20 mM NH_4OAc aq. with a flow rate of 0.75 mL/min, the radiotracer eluted within 65–71 min. The collected fractions were combined, diluted with water and adsorbed on a Sep-Pak C18 cartridge. The purified labeled product could be desorbed almost quantitatively with 1.5–2 mL MeOH. The final product was analyzed by analytical radio-HPLC and radio-TLC.

Method B: The MeCN eluate (2 mL) was diluted with 2 mL water and purified by preparative HPLC using a Reprosil 100 Phenyl column (250 × 20 mm ID, particle size 5 μm). The following parameters were applied: eluent 38% MeCN/water/15 mM AcOH/15 mM TEA; flow rate 8 mL/min. **[^{18}F]FBASV** eluted at t_R = 48–53 min well separated from the precursor (t_R = 55–60 min). The final product could be almost completely removed from the column (based on ^{18}F activity balance). The collected HPLC fractions were diluted with 50 mL water and transferred to a Sep-Pak C18 Plus cartridge. After trapping and washing with 10 mL water, **[^{18}F]FBASV** was almost quantitatively desorbed with 1.5–2 mL MeOH. For subsequent animal experiments, the solvent was evaporated and the radiotracer re-dissolved in EtOH or sterile isotonic saline.

Cartridge experiments

All cartridges were conditioned according to manufacturer's instructions. Crude labeling DMF mixtures were diluted with different amounts of water (50 mL for bare, 100 mL for C18, and 5 mL for cation exchanger cartridges). After sorption, the cartridges were air-dried and elution was performed with portions of solvents as described before. The main ^{18}F activity eluted with the first 1–2 mL of eluent.

In vitro stability and lipophilicity

The *in vitro* stability of **[^{18}F]FBASV** was tested by incubation (2 h at 40°C) of small tracer amounts (10–15 MBq; $\sim$250 μL) in 1 mL 0.9% NaCl, PBS (0.1 M Na_3PO_4, 0.15 M NaCl, pH 7.2), and TRIS–HCl (50 mM, pH 7.4).

Partition coefficients were determined with the shake-flask method in silicone-coated glass tubes. **[^{18}F]FBASV** was diluted in the respective buffer (20–50 μL, 1:1000) and added to a mixture of 3.0 mL of pre-equilibrated n-octanol and 3.0 mL of the following buffers: (i) phosphate buffer (50 mM KH_2PO_4/$Na_2HPO_4 \times 2H_2O$, pH 7.2), (ii) PBS (0.1 M Na_3PO_4, 0.15 M NaCl, pH 7.2), and (iii) TRIS-HCl (50 mM, pH 7.4). After shaking for 30 min at room temperature, the samples were centrifuged (4000*g*, 5 min) and the pH values were re-examined. Duplicates of samples (0.5 mL each) of the organic as well as the aqueous layers were measured in a gamma counter. Another duplicate of samples (1 mL each) of the organic layer were re-partitioned until constant partition coefficient values had been obtained. Measurements were done in triplicate.

Results and discussion

Chemistry and *in vitro* binding data

Based on the chemical structure of vesamicol, six novel fluoro-substituted azaspirovesamicol derivatives (**1a**–**3b**, structures in Table 1) were synthesized and studied regarding their *in vitro* binding data. The organic synthesis of these compounds has been described previously.[18,19] In brief, we utilized the method of nucleophilic ring opening of a 4-fluorobenzoyl substituted *syn*/*anti* epoxide mixture with 4-phenylpiperidine, which resulted in the formation of four different isomers (**1a**–**1d** in Figure 1).[23] **1a** and **1b** were isolated in satisfactory quantities via fractionated crystallization, whereas only small quantities of the other two isomers **1c** and **1d** could be isolated using semi-preparative HPLC. Because of similar R_f values of **1a**–**1d**, a separation via flash chromatography did not succeed. Therefore, we focused our efforts on the synthesis of compounds belonging to the regioisomeric groups **a** and **b**. Thus, the 4-fluorobenzyl (**2a**/**2b**) and 3-fluoropropyl (**3a**/**3b**)-substituted azaspirovesamicol derivatives were synthesized starting from **1a** and **1b** as previously described.[19]

The *in vitro* binding affinities of **1a**–**3b** to VAChT and to sigma-receptor sites were determined by competitive binding assays (K_i values in Table 1). The commercially available (−)-[^{3}H]vesamicol ((−)-[^{3}H]AH5183) was used as radioligand to

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Table 1. Structures and *in vitro* binding data of novel azaspirovesamicol derivatives **1a–3b**

Compound	Chemical structure	*R*	K_i (VAChT)	K_i ($\sigma_{1,2}$)	Selectivity factor
(±)-**1a**	HO, N, N–R; **1a = FBASV**	4-F-benzoyl	28.8±3.4	768±500	27
(±)-**2a**		4-F-benzyl	33.4±10.6	74.3±19.9	2.2
(±)-**3a**		3-F-propyl	61.1±19.1	753±278	12.3
(±)-**1b**	HO, N, N, R	4-F-benzoyl	1484±103	n.d.	/
(±)-**2b**		4-F-benzyl	1612±833	n.d.	/
(±)-**3b**		3-F-propyl	2882±803	854±199	0.3
(±)-vesamicol	HO, N		34.8±6.7	n.d.	/
(−)-vesamicol			11.3±1.6	121±49	11

K_i values in nM (mean±SD, $n>2$) were derived from IC_{50} values according to the equation: $K_i = IC_{50}/(1+C/K_D)$, where C is the concentration of the radioligand and K_D the dissociation constant of the corresponding radioligand; (−)-[^{3}H]vesamicol: $K_D = 3.7$ nM to VAChT on PC12 cells transfected with rat VAChT cDNA[22]; [^{3}H]DTG: $K_D = 39.8$ nM to $\sigma_{1,2}$ receptors on PC12 cells transfected with rat VAChT cDNA; n.d. means not determined; selectivity factor was calculated according to K_i(VAChT)/K_i($\sigma_{1,2}$).

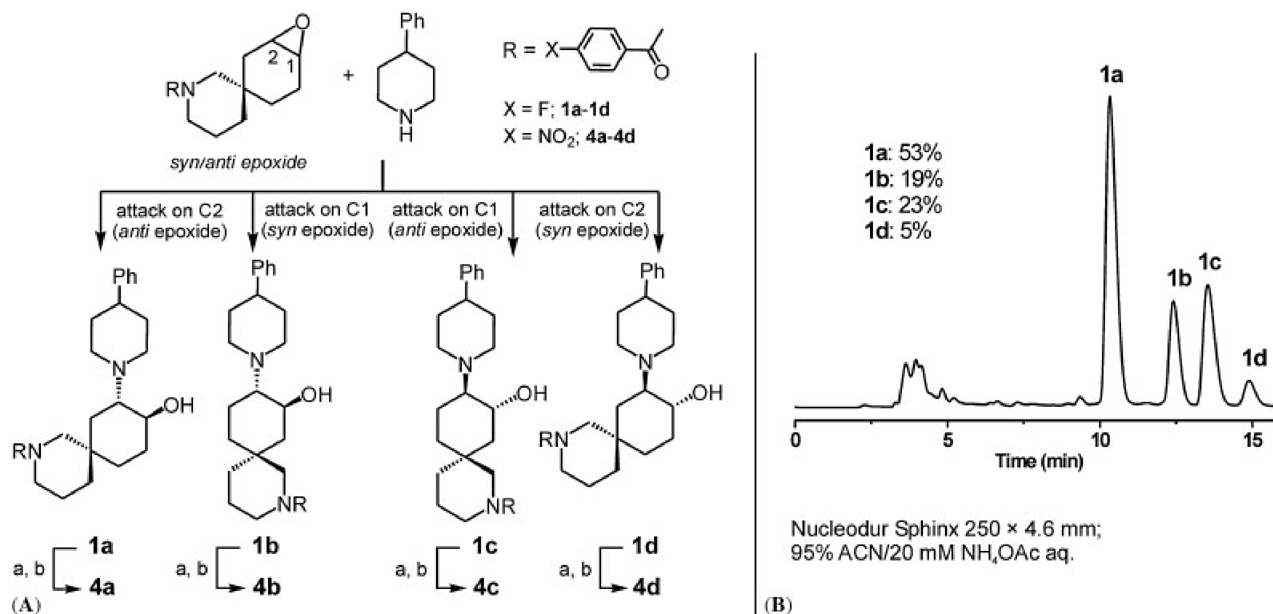

Figure 1. (**A**) Formation of the 4-fluorobenzoyl and 4-nitrobenzoyl substituted azaspirovesamicol isomers **1a–d** and **4a–d**: (a) 6M HCl and (b) nitrobenzoyl chloride/ $NaHCO_3$. (**B**) Analytical separation of **1a–d** by HPLC.

address VAChT binding sites. [^{3}H]DTG (1,3-di-o-tolylguanidine) was used to detect sigma binding of the compounds, because this radioligand displays high and roughly equal affinity for both σ_1 and σ_2 receptors.

Comparing the data obtained for the two structurally different groups **a** and **b**, derivatives of group **a** show VAChT affinities within the nanomolar range. By contrast, for the derivatives of group **b** K_i values $>1\,\mu$M were determined, which demonstrates the strong dependence of binding affinity on structural changes at this position of the molecule. Furthermore, while the substitution of the azaspiro[5.5]undecane ring system in para position to the OH functionality (group **a**) did not impair

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significantly the VAChT binding compared to (±)-vesamicol, the affinity to the sigma-receptors was decreased considerably (except for **2a**). Thus, a general increase of the target selectivity (defined as ratio of $K_i(\sigma_{1,2})/K_i(\text{VAChT})$) could be achieved with this chemical modification of the vesamicol skeletal structure. In particular, **1a** showed the most pronounced 2.5-fold increase of the selectivity factor.

As the 4-fluorobenzoyl derivative **1a** (**FBASV**) demonstrated the highest affinity towards VAChT *in vitro* and an improved selectivity of VAChT binding over sigma receptor binding, this compound was selected for radiofluorination and future *in vivo* evaluation.

For ^{18}F-labeling of **FBASV** the corresponding nitro-precursor **4a** was synthesized by starting with the isomeric mixture **1a**–**1d**, which was firstly debenzoylated under acidic conditions to get the secondary amines. In a second step, the 4-nitrobenzoyl group was introduced using 4-nitrobenzoyl chloride under basic conditions to get the isomeric nitro-precursor mixture of **4a**–**4d** (A in Figure 1). The isolation of isomeric pure **4a** was accomplished via semi-preparative HPLC using a Nucleodur Sphinx column. A separation of **4a** using flash chromatography (silica and alumina) was not successful due to very similar R_f values of the four isomers.

Radiochemistry

The radioligand **[^{18}F]FBASV** was prepared by nucleophilic aromatic substitution of the nitro precursor **4a** using anhydrous [^{18}F]KF-K2.2.2-carbonate complex in DMF (Figure 2).

Initial labeling experiments using dimethylformamide (DMF) with conventional heating at various temperatures failed. As the use of microwaves is often beneficial for the nucleophilic substitution of a nitro group by [^{18}F]fluoride,[15,24] we performed the labeling procedure by utilizing this technique. At defined conditions (75 W, 154–156°C, 25 min) reproducible labeling yields of 25–30% were achieved.

Within 30 min of reaction time, the labeling yield increased continuously. Under these reaction conditions the nitro precursor **4a** did not show considerable decomposition. Using radio-HPLC and radio-TLC, besides [^{18}F]F$^-$ only a single highly polar radioactive by-product was observed, whose structure was not identified. The labeling yield was found to depend on the amount of nitro precursor. Best labeling yields (25–30%) were achieved with precursor amounts of 3.0–3.5 mg.

For purification of the radiotracer via semi-preparative HPLC initial analytical HPLC studies were performed. The separation of **[^{18}F]FBASV** and its nitro precursor showed difficulties arising from the similarity in their chromatographic behavior. This is occasionally observed when nitro precursors are used.[15,24,25] Furthermore, it is beneficial when the labeled compound has a retention time shorter than the precursor; otherwise it is difficult to avoid contamination of the tracer due to the bulk of remaining precursor. Therefore, two alternative approaches for the isolation of **[^{18}F]FBASV** were developed: Method A is based on the reduction of excess nitro precursor to the corresponding amine followed by semi-preparative HPLC separation on a common RP18 column. For method B we accomplished a comprehensive HPLC study to find a suitable column for the direct separation of **[^{18}F]FBASV** and its nitro precursor.

Method A

To change the chromatographic properties of the compounds to be separated, the remaining nitro precursor was reduced to its corresponding amine according to a procedure which was successfully applied in our group.[15,26] In brief, after pre-purification of the reaction mixture on an RP cartridge the reduction was accomplished using palladium on barium sulfate as catalyst in the presence of ammonium formate in methanol (MeOH). In this process a significant quantity of ^{18}F activity was adsorbed on the palladium surface, which could be attributed to the highly polar by-product. On a second RP cartridge the solvent was changed from MeOH to acetonitrile (MeCN) and the desired product was isolated by semi-preparative HPLC using a Multospher RP18 AQ column (previous attempts to separate the nitro precursor **4a** from the labeled compound with this column were not successful). Because of the higher polarity of the amine it elutes in front of **[^{18}F]FBASV** (Figure 4). However, due to the high concentration of this reduced precursor compound, a very broad peak was observed. In order to assure a high purity of the radiotracer, the flow rate has to be reduced resulting in a long retention time of the labeled product. Finally, **[^{18}F]FBASV** could be prepared in 10–12% RCY, with a specific activity ranging from 50 to 100 GBq/μmol, and a radiochemical purity of ⩾99.0% at a total synthesis time of about 3.5 h (conditions not fully optimized).

Method B

The main aim of this approach was to avoid the reduction step of **4a** to reduce the total radiosynthesis time. Therefore, we accomplished an analytical HPLC study with **FBASV** and **4a** to find a suitable column for the direct preparative separation of **[^{18}F]FBASV**, at which the labeled compound elutes in front of the nitro precursor **4a**.[27] A phenyl column was found to be most appropriate for this separation using MeCN/water and 15 mM triethyl amine (TEA)/15 mM acetic acid (AcOH) as buffer. Compared to the NH_4OAc buffer system, the use of TEA/AcOH reduced both peak tailing as well as retention times and improved the separation factor α (Figure 3). By the application of MeOH as organic modifier the separation of the two compounds did not succeed, neither with NH_4OAc nor with TEA/AcOH as buffer.

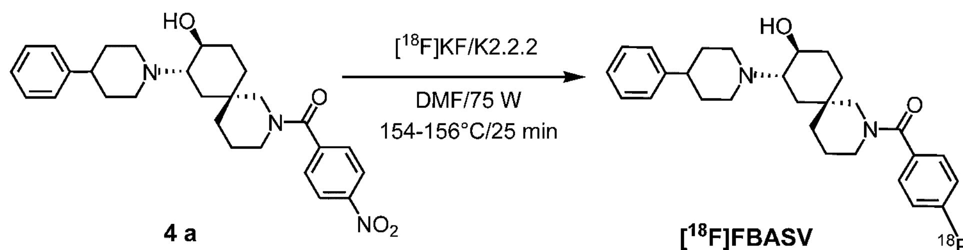

Figure 2. Radiosynthesis of **[^{18}F]FBASV**.

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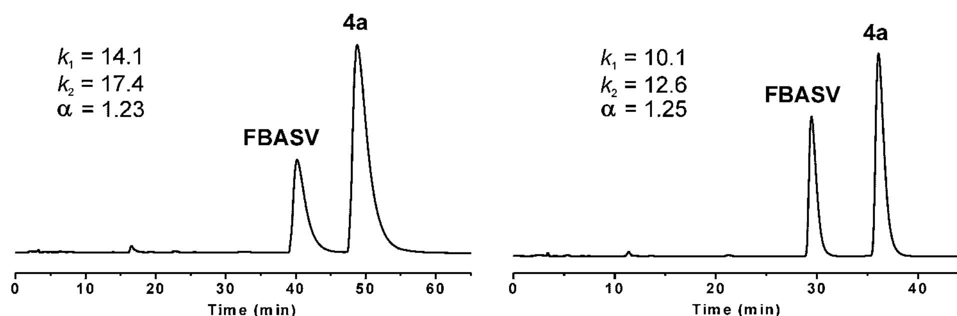

Figure 3. Analytical separation of **FBASV** and **4a** using a Reprosil 100 Phenyl column; left: 35% MeCN/20 mM NH_4OAc aq., 1 mL/min; right: 32% MeCN/water/15 mM TEA/15 mM AcOH, 1 mL/min.

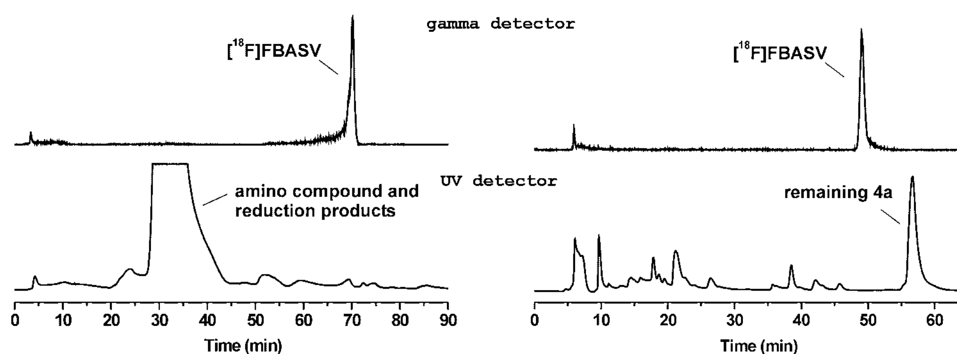

Figure 4. *Left*: Semi-preparative HPLC separation of **[^{18}F]FBASV** after total reduction of the excess precursor according to method A (Multospher 120 RP AQ 150 × 10 mm ID, 52% MeCN/20 mM NH_4OAc aq., 0.75 mL/min, injection volume 4 mL). *Right*: Preparative HPLC separation of **[^{18}F]FBASV** without reduction of the excess precursor according to method B (Reprosil 100 Phenyl 250 × 20 mm ID, 38% MeCN/water/15 mM AcOH/15 mM TEA, 8 mL/min, injection volume 4 mL).

The analytical data were transferred to the preparative separation of **[^{18}F]FBASV** (Figure 4), as a result of which the total radiosynthesis time could be decreased with method B from 3.5 h (method A) to 2.5 h. Furthermore, an improved RCY of 16–18%, a specific activity >300 GBq/μmol, and a radiochemical purity of >99.5% could be achieved.

Additional cartridge experiments

A series of experiments was performed using other than C18 cartridges to verify the need of a semi-preparative HPLC separation of **[^{18}F]FBASV**. Since the radiotracer is a protonizable tertiary amine, acidic cation exchangers based on benzene-sulfonic acid derivatives were tested initially (Chromafix SA and Oasis® MCX as mixed-mode cation-exchange and reversed-phase sorbent especially for bases). Starting with highly diluted, crude labeling DMF solutions, 35–50% of the radioactive substances were bound. This is slightly more than the labeling yield, because also the polar by-product was adsorbed. Most of the by-product could be removed from cartridge by elution with 0.1 M H_3PO_4 or 0.5 M aq. NaCl. Subsequently, the elution of the radiotracer was nearly quantitative with alkalescent eluents such as 5% NH_3/MeOH or EtOH and 1% TEA/EtOH. However, the radiochemical purity of the eluate was only 80–90% and the precursor was not separated completely.

Furthermore, the two bare cartridge materials aluminum oxide (ALOX N) and silica (Sep-Pak® Plus Silica) were tested. With the ALOX N cartridge, [^{18}F]F^- could be removed completely from reaction mixture; however, **[^{18}F]FBASV** and the precursor were not separated sufficiently. Using the silica cartridge, the radiotracer could not be eluted with high radiochemical purity.

Finally, we can conclude that an isolation of **[^{18}F]FBASV** with high chemical and radiochemical purity was not possible using these special SPE cartridges.

In vitro stability and lipophilicity of [^{18}F]FBASV

Chemical stability of the purified radiotracer **[^{18}F]FBASV** was investigated by incubation at 40°C in following solutions: (i) 0.9% aq. NaCl, (ii) phosphate-buffered saline (PBS, pH = 7.2), and (iii) TRIS-HCl (pH = 7.4). At various time points, aliquots were analyzed by radio-TLC and radio-HPLC. **[^{18}F]FBASV** proved to be stable in all media, and no defluorination or degradation was observed within 2 h.

To estimate the lipophilicity of **[^{18}F]FBASV**, *n*-octanol-water partition coefficients (log *D* at pH = 7.2–7.4) were experimentally determined by repeated multiple extraction (shake-flask method). Use of three conventional buffers resulted in log *D* values of 1.96 ± 0.83 (*n*-octanol/PBS at pH 7.2), 2.10 ± 0.66 (*n*-octanol/TRIS-HCl at pH 7.4), and 2.12 ± 0.19 (*n*-octanol/phophate buffer at pH 7.2), respectively. With these values the radiotracer demonstrates a moderate lipophilicity, which should result in a sufficient permeation of the blood–brain barrier *in vivo*.

Summary/conclusion

Six novel azaspirovesamicol analogs (**1a**–**3b**) were characterized regarding their *in vitro* binding toward VAChT and to sigma receptors, the latter as criterion for the selectivity of the ligand binding. Because of its appropriate binding data, the 4-fluorobenzoyl substituted derivative **FBASV** was selected as candidate for ^{18}F-labeling. **[^{18}F]FBASV** was prepared by

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microwave assisted nucleophilic aromatic substitution of the corresponding 4-NO_2-benzoyl precursor. As the chromatographic separation of the labeled compound and its nitro precursor proved to be difficult, two radiosynthetic approaches were developed. Utilizing method A, the remaining nitro precursor was reduced in an additional step to its corresponding amine, which could be separated via semi-preparative HPLC on a conventional RP column. As the highly concentrated precursor eluted in front of the labeled compound, long retention times were needed to separate **[^{18}F]FBASV**. To improve the radiosynthetic procedure and to shorten the total radiosynthesis time, a direct HPLC separation of the radiotracer and its nitro precursor was intended. A phenyl column was found to be most suitable for this purpose (method B). With this column a change of the elution order could be achieved, which is beneficial for a proper separation. Because of the reduced number of radiosynthetic steps, with method B the total radiosynthesis time could be decreased from 3.5 h (method A) to 2.5 h. Furthermore, an increased RCY of 16–18%, a specific activity $>$300 GBq/μmol, and a radiochemical purity of $>$99.5% could be achieved.

With this improved HPLC separation of **[^{18}F]FBASV** and its nitro precursor, a reliable and efficient radiosynthesis of **[^{18}F]FBASV** has been developed, suitable for the production of sufficient quantities of the radiotracer for future investigation *in vivo*.

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