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Synthesis, Biodistribution and PET Studies in Rats of ^{18}F-Labeled Bridgehead Fluoromethyl Analogues of WAY-100635

Rana Al Hussainy[§], Joost Verbeek[§], Dion van der Born[§], Carla Molthoff[§], Jan Booij[¥] and J(Koos) D.M. Herscheid[§]

[§]*Department of Nuclear Medicine & PET Research, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands.*
[¥]*Department of Nuclear Medicine, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.*

Abstract

In vitro screening of fluoromethyl bridge-fused ring (BFR) analogues of WAY-100635 (**5a**, **5b** and **5c**) has shown a high binding affinity and a good selectivity for the 5-HT$_{1A}$ receptor. As these compounds were designed to provide PET ligands with high metabolic stability, they are now radiolabeled with fluorine-18 and investigated *in vivo*. BFR precursors were synthesized and reacted with fluorine-18 in dry MeCN in the presence of 2,2,2-kryptofix and K$_2$CO$_3$ as catalysts under standard conditions. In rats, bio -distribution and PET studies were performed using [^{18}F]**5a**, [^{18}F]**5b** and [^{18}F]**5c**. The binding specificity was determined by administration of non-labeled WAY-100635 prior to the radiolabeled ligands. [^{18}F]**5** ligands were synthesized in overall radiochemical yields of 24-45%, respectively with a radiochemical purity of >98%. Relatively good hippocampus to cerebellum ratios of 5.55, 4.79 and 5.45, respectively were reached at 45 minutes after injection. However, PET studies indicated defluorination of the radioligands by showing high accumulation of radioactivity in the bones in the order of [^{18}F]**5a** ≈ [^{18}F]**5b** > [^{18}F]**5c**.

In conclusion, the radioligands bind preferentially to the 5-HT$_{1A}$ receptor *in vivo*. Unfortunately, no metabolic stability with regard to defluorination was observed.

Introduction

The importance of the neurotransmitter serotonin (5-HT) in the pathophysiology of anxiety is well known.[1,2] Also, imaging studies of the neuronal activity and the volume of 5-HT_{1A} receptor in the brains of depressed patients were investigated[1,2,3,4] as well as patients with Alzheimer's disease (AD)[5,6] and schizophrenia[7,8,9]. In normal human brain, high 5-HT_{1A} receptor concentrations are found in the brainstem raphe nuclei and high densities of postsynaptic 5-HT_{1A} receptors are found in the limbic forebrain (hippocampus, entorhinal cortex and septum). Low densities are observed in the basal ganglia and in the adult cerebellum.[1]

For positron emission tomographic (PET) imaging of these receptors, [carbonyl-^{11}C]WAY-100635 is the "gold standard".[10,11,12,13] Although the amide bond is rapidly hydrolyzed *in vivo*, the resulting radioactive metabolite does not enter the brain and as such enables proper quantification of 5-HT_{1A} receptor parameters. However, the introduction of the ^{11}C-label requires a very complex reaction sequence; even an optimized automated one-pot synthesis gives a failure rate of nearly 10%.[14] Furthermore, due to the short physical half-life of 20 minutes, this radioligand is only available for hospitals having an in-house cyclotron and radiopharmacy. Taking this into consideration, studies have been directed toward ^{18}F-labeled analogues of WAY-100635 (Figure 1).
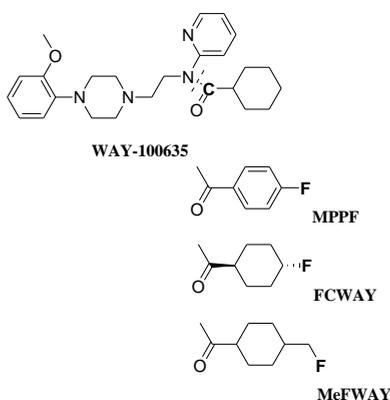

Figure 1. WAY-100635 and WAY-100635 PET analogues; *p*-MPPF, FCWAY, and MeFWAY.

The 5-HT$_{1A}$ antagonist [^{18}F]MPPF was found to be prepared easily, selective and with high affinity for the 5-HT$_{1A}$ receptor, but lower than WAY-100635.[15] Pre-treatment with non-labeled WAY-100635 showed only 60% reduction of brain uptake.[16] Unfortunately, this radiotracer seems to be a substrate for P-glycoprotein (P-gp)[17]– probably due to the extra aromatic group[18]– implicating a relatively low amount reaching the brain. To circumvent the additional aromatic moiety, a variety of fluorocyclohexane derivatives (FCWAYs) have been synthesized and evaluated showing the best properties for *trans*-4-FCWAY[19] to measure 5-HT$_{1A}$ receptor density. However, the synthesis of the corresponding ^{18}F-labeled compound is hampered by the formation of difficult to separate non-radioactive contaminants[20] such as the elimination product, which may act as a "pseudo-carrier", but the major limitation is its *in vivo* defluorination. Placing a fluorine on a primary carbon as in [^{18}F]MeFWAY greatly reduced this *in vivo* instability.[21]

Recently, we reported the synthesis and the *in vitro* screening of bridge-fused ring (BFR) analogues of WAY-100635 with a fluoromethyl group on the bridge-head, i.e. the non-labeled form of **5a**, **5b** and **5c** (Figure 2). These analogues showed a high binding affinity (K_i = 0.86, 0.54 and 0.65 nM, respectively) and a good selectivity for the 5-HT$_{1A}$ receptor over other related receptors.[22] Furthermore, it was found that BFR analogues are more stable toward amide hydrolysis than [^{18}F]MPPF.[22,23] The aim of this study is to evaluate if the ^{18}F-labeled **5a**, **5b** and **5c** could serve as suitable PET ligands, circumventing the above mentioned disadvantages of the currently clinically used [carbonyl-^{11}C]WAY-100635, [^{18}F]MPPF and [^{18}F]FCWAY. In this chapter, the synthesis of these new radioligands, as well as their biodistribution and PET studies in rats are described.
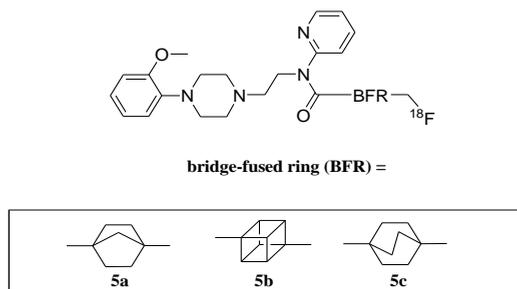

Figure 2. Fluoromethyl BFR analogues of WAY-100635.

Results

Chemistry

A possible general route to the needed precursors **4** is depicted in Scheme 1. Formation of the acid chlorides of **1** was achieved with thionyl chloride in MeCN, which in all cases could effectively been coupled to WAY-100634 to give **2**. Reduction of the ester group with NaBH$_4$-LiCl was surprisingly only applicable for the synthesis of **3a** and **3b**.

a, BFR = Bicyclo[2.2.1]heptane
b, BFR = Cubane
c, BFR = Bicyclo[2.2.2]octane
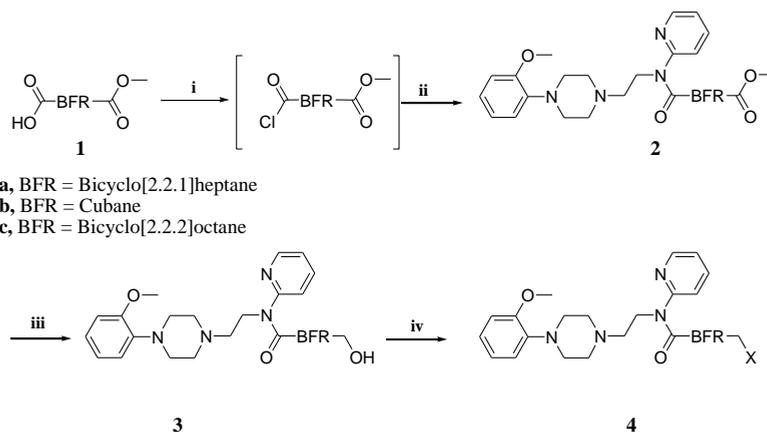
Scheme 1. General synthetic pathway for the precursors **4**: (i) thionyl chloride, MeCN, reflux; (ii) WAY-100634, NEt$_3$, MeCN, RT.; (iii) NaBH$_4$, LiCl, 1-methoxy-2-(2-methoxyethoxy)ethane, reflux; (iv) for BFR = bicyclo[2.2.1]heptane; 4-methylbenzene-1-sulfonyl chloride, pyridine, CH$_2$Cl$_2$, RT., for BFR = cubane; triphenyl phosphine, CBr$_4$, CH$_2$Cl$_2$, 0 $^{\circ}$C

Conversion of **2c** to **3c** was found to be non-selective leading to a reduction of the amide bond too. The primary alcohols (**3a** and **3b**) needed to be converted into a good leaving group (LG) for the subsequent fluorination step. The tosylate **4a** was synthesized in high yield (82%) under standard reaction conditions. As expected, tosylation of **3b** resulted in a ring enlargement leading to the homocubyl derivative.[22] Therefore, **3b** was converted into the bromide **4b** in moderate yield (49%) which was found to be stable when kept at ambient temperature.
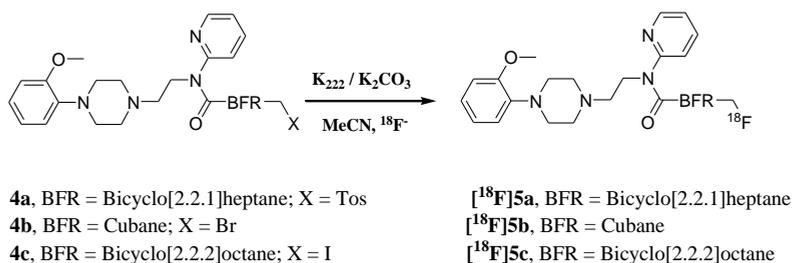
Since a selective reduction of **2c** or its free acid to **3c** was unsuccessful, **4c** was synthesized by the route shown in Scheme 2. Compound **6** was obtained by the reduction of **1c** with borane dimethyl sulfide and subsequently converted to **7**. Since it was impossible to saponify **7**, this compound was first converted into **8** using NaI followed by saponification to give **9**. To prevent chloro-deiodination by thionyl chloride, the acid chloride of **9** was obtained using dichloro(methoxy)methane[24] in CH$_2$Cl$_2$ and was then coupled to WAY-100634 to afford **4c** in good yield (63%).
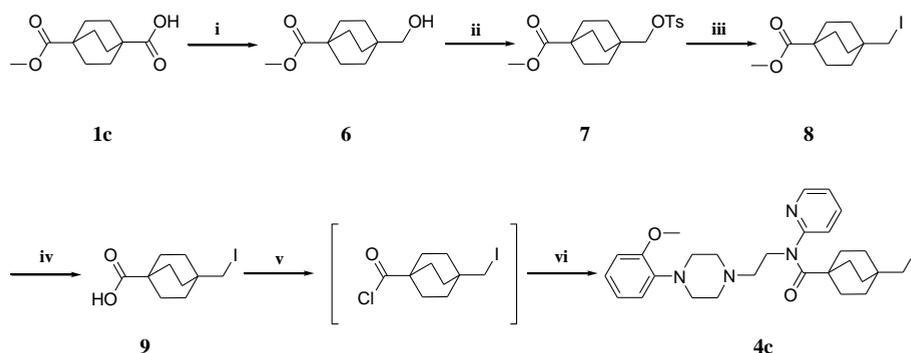
Scheme 2. Synthetic pathway for the precursor **4c**: (i) BH$_3$S(CH$_3$)$_2$, THF, 0 ^{o}C-RT.; (ii) 4-methylbenzene-1-sulfonyl chloride, pyridine, RT.; (iii) NaI, 2-methoxyethanol, 130 ^{o}C; (iv) NaOH, MeOH:H$_2$O (10:1), RT.; (v) dichloro(methoxy)methane, CH$_2$Cl$_2$, 60 ^{o}C; (vi) WAY-100634, NEt$_3$, CH$_2$Cl$_2$, RT.

Radiolabeling

A general synthetic route to the desired radiofluorinated analogues of WAY-100635 is depicted in Scheme 3.

4a, BFR = Bicyclo[2.2.1]heptane; X = Tos
4b, BFR = Cubane; X = Br
4c, BFR = Bicyclo[2.2.2]octane; X = I

[^{18}F]**5a**, BFR = Bicyclo[2.2.1]heptane
[^{18}F]**5b**, BFR = Cubane
[^{18}F]**5c**, BFR = Bicyclo[2.2.2]octane

Scheme 3. Radiosynthesis of [^{18}F]**5a**, [^{18}F]**5b** and [^{18}F]**5c**.

Radiofluorination of **4** using 2,2,2-kryptofix/K_2CO_3 in acetonitrile proceeded without extensive decomposition of the precursor. As an example, the radiolabeling of **4c** for which the highest reaction temperature (150 ^{o}C) was needed is shown in Figure 3. Conversion of **4b** proceeded already at 110 ^{o}C, presumably due to the pseudo-aromatic character of the cubyl moiety. Preparative HPLC afforded [^{18}F]**5** in fair to good overall radiochemical yields (24-45%) with acceptable SA's (76-138 GBq/µL). Analytical HPLC revealed the presence of only very small amounts of unlabeled contaminants which are believed to hardly influence the biodistribution of [^{18}F]**5**.
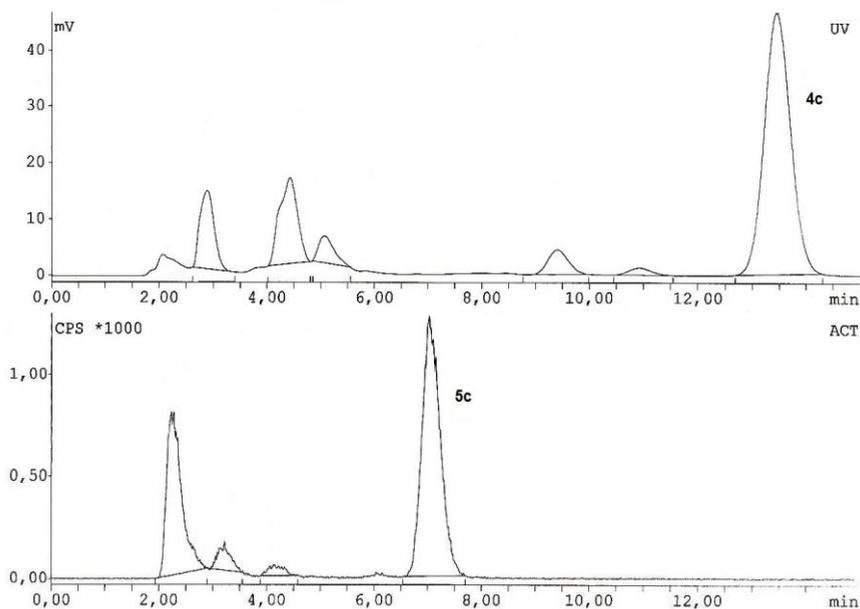

Figure 3. HPLC chromatogram of crude reaction mixture of [^{18}F]**5c** (kromasil C18-10 µm column, 4.6 mm x 250 mm); eluent (MeOH/H_2O/DIPA, 80:20:0.02).

Biodistribution of [^{18}F]5a, [^{18}F]5b and [^{18}F]5c in rat

The results of the biodistribution of [^{18}F]**5a**, [^{18}F]**5b** and [^{18}F]**5c** are shown in Table 1.

Table 1. Uptake of [^{18}F]**5a**, [^{18}F]**5b** and [^{18}F]**5c** in rats at 45 min p.i.[a]

	[^{18}F]5a	[^{18}F]5b	[^{18}F]5c
Blood	0.13 ± 0.02	0.16 ± 0.00	0.13 ± 0.03
Heart	0.09 ± 0.01	0.09 ± 0.00	0.13 ± 0.01
Lung	0.18 ± 0.07	0.14 ± 0.01	0.21 ± 0.01
Liver	0.62 ± 0.02	1.34 ± 0.06	0.76 ± 0.06
Kidney	0.45 ± 0.04	0.55 ± 0.01	0.46 ± 0.01
Frontal cortex	0.06 ± 0.01	0.06 ± 0.01	0.09 ± 0.01
Occipital cortex	0.06 ± 0.02	0.07 ± 0.01	0.15 ± 0.03
Striatum	0.04 ± 0.00	0.03 ± 0.01	0.05 ± 0.01
Hippocampus	0.17 ± 0.01	0.16 ± 0.01	0.24 ± 0.03
Cerebellum	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.01

[a] Values are averaged ($n = 4$) %ID/g ± SD and decay corrected.

The degree of binding specificity in a brain area is often expressed as the ratio of radioactivity uptake in that area over the cerebellum, taken as a receptor-devoid reference tissue (Table 2).

Table 2. The ratio of uptake of [^{18}F]**5a**, [^{18}F]**5b** and [^{18}F]**5c** in 5-HT$_{1A}$-rich brain regions over uptake in the cerebellum[a].

Region of interest	[^{18}F]5a	[^{18}F]5b	[^{18}F]5c
Hippocampus	5.55 ± 0.91	4.79 ± 0.37[*]	5.45 ± 0.31[*]
Occipital cortex	1.89 ± 0.31	2.18 ± 0.31[*]	3.36 ± 0.50
Frontal cortex	1.69 ± 0.01	1.66 ± 0.21[**]	2.14 ± 0.08[**]
Striatum	1.20 ± 0.27	0.98 ± 0.17[**]	1.09 ± 0.30[**]

[a]Results are presented as ratios ± SD (45 min after injection; $n = 4$) of uptake in specific brain regions over cerebellum [*]$P \leq 0.01$,[**]$P \leq 0.05$.

PET studies

The brain uptake in time and the specific binding of the novel radioligands were determined using PET. As in the biodistribution study, brain uptake of [^{18}F]**5a** and [^{18}F]**5b** was almost the same, while that for [^{18}F]**5c** was clearly higher (Figure 4).
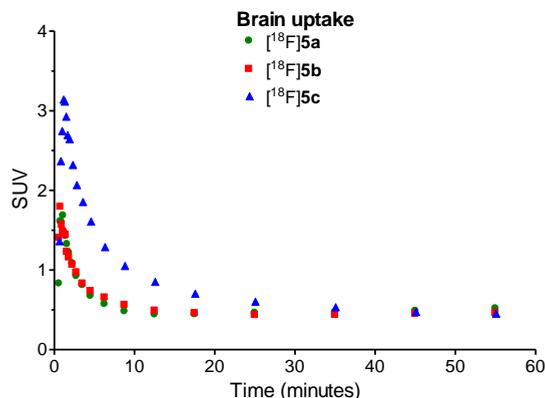

Figure 4. PET data of brain uptake of ^{18}F radioligands.

Pre-treatment with WAY-100635 showed for all three radioligands an increased washout from the brain (for an example see Figure 5). This indication of specific binding was confirmed by dissection of the rats directly after the PET study (60 minutes after injection). The hippocampus over cerebellum ratios of the non-pre-treated rats were slightly lower than those at 45 minutes ([^{18}F]**5a**: 3.72, [^{18}F]**5b**: 2.06 and [^{18}F]**5c**: 4.56) but after pre-injection with WAY-100635 all ratios went down to 1.
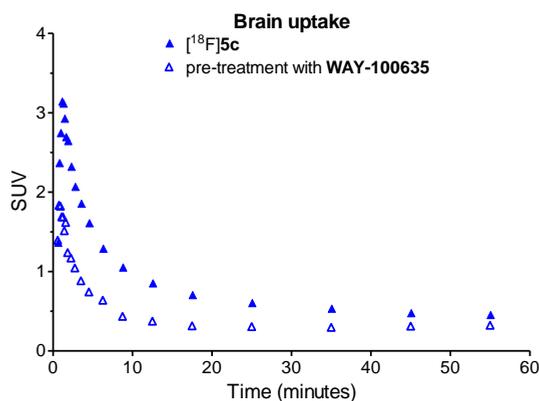

Figure 5. PET data of brain uptake of [^{18}F]**5c** without and with pretreatment with WAY-100635.

However, at the end of the study radioactivity uptake in the skeleton was visible (Figure 6), indicating that all three radioligands defluorinated *in vivo*.

Figure 6. Cumulative uptake of ^{18}F radioactivity at the end of the PET study.

The rate of bone uptake of radioactivity and thus the order of defluorination is [^{18}F]**5a** ≈ [^{18}F]**5b** > [^{18}F]**5c** (Figure 7).
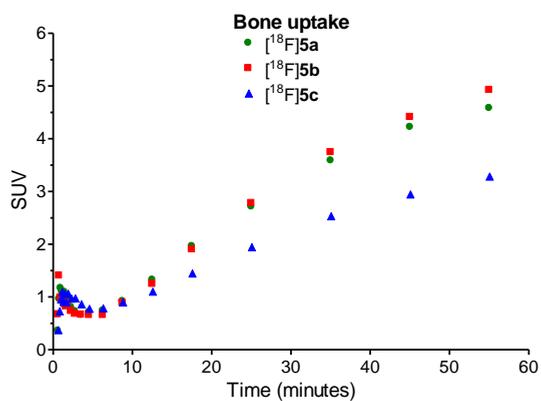

Figure 7. PET data of the [^{18}F]fluoride accumulation in bone.

Discussion

All the currently clinically used radiopharmaceuticals for imaging the 5-HT$_{1A}$ receptor have some disadvantages. Thus, there is still a strong need for an effective PET ligand for this receptor preferentially labeled with fluorine-18. Analogues containing the moiety WAY-100634 have a good chance to display high 5-HT$_{1A}$ receptor binding affinity. However, this moiety should not contain the radiolabel as it also enters the brain interfering with the scintigraphic measurements.[19,25,26] Fluoromethyl-BFR-analogues were found to have a high affinity and good selectivity for the target 5-HT$_{1A}$ receptor, at least comparable to that of WAY-100635.[22] Since the three analogues **5a**, **5b** and **5c** showed lipophilicities (log $D_{7.4}$ = 2.70, 2.94 and 3.37, respectively) that are close to that of WAY-100635 (log $D_{7.4}$ = 3.03)[22] and they all fall within the range log $D_{7.4}$ = 2-3.5[27], it is likely that these analogues will also penetrate the BBB and reach the target receptor in the brain. For a full *in vivo* evaluation it was decided to synthesize ^{18}F-labeled **5a**, **5b** and **5c**.

The synthesis route of precoursors **4a** and **4b** (Scheme 1) was not valid for **4c** (Scheme 2). Reduction of the ester group of **2c** with NaBH$_4$-LiCl found to be non-selective leading to a reduction of the amide bond too. The reason for this is not fully clear but might be caused by the difference in S-character of the exocyclic bond of the BFR's. The calculated hybridization for cubane is sp$^{2.2}$ compared to sp$^{2.8}$ for bicyclo[2.2.1]heptane and sp$^{3.2}$ for bicyclo[2.2.2]octane.[28,29]

As expected, a nucleophilic radiofluorination of **4** as shown in Scheme 3, where X is a good leaving group, was preceded cleanly and in fair to good overall radiochemical yields. Elimination of HX - which often occurs under the alkaline reaction conditions-, is herein not possible since HX is placed on a primary carbon, facilitating an easy HPLC separation.

In rats, all three radioligands ([^{18}F]**5a**, [^{18}F]**5b** or [^{18}F]**5c**) showed a higher uptake in the 5-HT$_{1A}$ receptor containing regions (hippocampus and cortex) than in devoid regions (cerebellum and striatum) (P <0.01, Table 1). The uptake of [^{18}F]**5a** and [^{18}F]**5b** was almost identical except for a higher uptake in the liver, which might be due to the enhanced hepatic activity for the cubyl moiety in [^{18}F]**5b**.[30,31] The uptake of [^{18}F]**5c** was higher than the uptake of [^{18}F]**5a** as well as of [^{18}F]**5b** in all brain tissues but not in the peripheral organs. The ratios of hippocampus over the cerebellum (Table 2) were higher

than those for [^{18}F]*p*-MPPF (hippocampus; 3, frontal cortex; 1.4 and striatum; 0.93)[32], while similar ratios were observed regarding frontal cortex and striatum. The observed ratios were lower than those reported for WAY-100635 (hippocampus; 14.3, frontal cortex; 7.7, and striatum; 1.6)[12]. These low ratios of the novel radioligands compared to the WAY-100635 could be due to a non-specific binding in the cerebellum, however, a recent *in vitro* study suggests that the high ratios of WAY-100635 might also be due to its high binding affinity to the dopamine D_4 receptor.[33]

As shown in Figures 6 and 7, these radioligands were defluorinated *in vivo*. The rate of bone uptake of radioactivity [^{18}F]**5a** and [^{18}F]**5b** was almost identical. The rate of bone uptake of radioactivity of [^{18}F]**5c** was lower than that of [^{18}F]**5a** as well as [^{18}F]**5b**. This was rather disappointing since these ligands were designed as the first WAY-100635 analogues, labeled with fluorine-18 on a primary carbon atom attached to the bridgehead of a BFR whereby HF elimination is chemically impossible. There are several enzymes by which fluorine containing ligands can be metabolized such as cytochrome P450 isozyme 2E1 (CYP2E1) and glutathione S-transferase (GST).[34] CYP2E1 might be responsible for an α-hydroxylation at the carbon to which fluorine is attached followed by an elimination of hydrofluoric acid, which will yield an aldehyde. In this case, defluorination can be reduced by pre-administration of CYP2E1 inhibitors like miconazol[35] or disulfiram[26]. Since a stability study of [^{18}F]**5b** in human hepatocytes[22], which should also contain CYP2E1, did not reveal the formation of free fluoride, this isozyme is probably not involved. However, α-hydroxylation by another enzyme cannot be excluded. GST is known to dehalogenate mostly alkyl iodines, bromides, and chlorides by nucleophilic substitution. This has recently also been suggested for the CH_2F in SP203. GST shows species and tissue variations in its activity and is significantly lower in human subjects than in rats.[36] In that case, [^{18}F]**5** ligands might be stable in human with regard to defluorination. It would be interesting, and for getting permission to study these radioligands in human even essential, to elucidate which of the above enzymes is responsible for the release of [^{18}F]fluoride from the radioligands, or if there is maybe another pathway involved.

Although the present study has shown that a rational design is not always a guarantee for success, at least in rats, we are currently focusing on derivatives with a CF_3

group instead of the CH_2F and on analogues which have the fluoro atom directly attached to the bridgehead. In both α-hydroxylation is no longer possible whereas in the last also a nucleophilic attack by e.g. glutathione is precluded while HF elimination remains unlikely because this would lead to a highly strained ring system.

Conclusion

Three ^{18}F-labeled radioligands, chemically stable against HF elimination, have been prepared in good radiochemical yields. They all showed a specific binding to the 5-HT_{1A} receptor in rat brain. Unfortunately, these compounds appeared to be unstable toward *in vivo* enzymatic defluorination in rats, which suggest that they are not better radioligands than the already clinically used WAY-100635 analogues.

Experimental section

Chemistry: General procedures.

4-Methoxycarbonylcubanecarboxylic acid was purchased from Boron Molecular (North Caroline, USA). 4-(Methoxycarbonyl)bicyclo[2.2.2]octane-1-carboxylic acid was purchased from Atlantic research chemicals Ltd (Bude, UK). All other chemicals used were reagent grade and were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Citrate buffer (pH 4.8-5.0) was made in house (480 mg citric acid trisodium salt dihydrate, 240 mg citric acid and 210 mg sodium acetate in 30 mL water). SepPak C18 plus cartridges and SepPak tC18 plus cartridges were obtained from Waters Chromatography B.V. (Etten-Leur, The Netherlands). [18]F (nca.) in $[^{18}O]H_2O$ was produced by BV Cyclotron VU using proton bombardment on 99.9% enriched [18]O. Nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR) were determined in the indicated solvent using a Bruker AC 200 MHz (200.13 MHz and 50.32 MHz, respectively) or a Bruker Avance 250 MHz (250.13 and 62.90 MHz). Chemical shifts (δ) are given in ppm downfield from tetramethylsilane (^{1}H, ^{13}C) and coupling constants (*J*) in Hz. High performance liquid chromatography (HPLC) was carried out using a kromasil C18 column (4.6 mm x 250 mm). Peaks were detected at 254 nm. The radioactivity of the eluate was monitored using an inline NaI(Tl) radiodetector.

Synthesis of the precursors

Methyl4-(N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)carbamo yl) bicyclo[2.2.1]heptane-1-carboxylate (2a).

4-(methoxycarbonyl)bicyclo[2.2.1]heptane-1-carboxylic acid (**1a**) was synthe-sized as mentioned previously.[22] In a flame dried flask under argon atmosphere, compound **1a** (353 mg, 1.79 mmol) was dissolved in MeCN (10 mL) and thionyl chloride (3 mL) was added. The reaction mixture was refluxed at 70 ^{o}C for 1 hour, the excess of thionyl chloride was totally evaporated under reduced pressure and coevaporated with MeCN (3 x 3 mL). To the corresponding acid chloride, WAY-100634 (564 mg, 1.81 mmol) and NEt$_3$ (247 μL) in MeCN (10 mL) were added and stirred at room temperature overnight. The solvent was evaporated, the residue was dissolved in water and extracted with CH$_2$Cl$_2$ (3 x 10 mL). The organic phase was collected, dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness. Column chromatography (EtOAc/NEt$_3$, 1:0.01) gave **2a**; yield: 803 mg (90%) as a white solid. ^{1}H NMR (250.13 MHz, CDCl$_3$): δ 1.08-1.30 (m, 2H, CH$_2$), 1.40-1.60 (m, 2H, CH$_2$), 1.70-2.00 (m, 6H, 3 x CH$_2$), 2.48-2.70 (m, 6H, N(CH$_2$)$_3$), 2.80-3.10 (m, 4H, N(CH$_2$)$_2$), 3.60 (s, 3H, CH$_3$), 3.70-4.00 (m, 5H, NCH$_2$ and CH$_3$), 6.75-7.00 (m, 5H, 5 x CH), 7.22-7.30 (m, 1H, CH), 7.70 (dt, J = 7.6 Hz, 1H, CH), 8.54 (d, J = 4.9 Hz, 1H, CH).

4-(Hydroxymethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl) bicyclo[2.2.1]heptane-1-carboxamide (3a).

In a flame dried flask under argon atmosphere, compound **2a** (366 mg, 0.74 mmol) was dissolved in 1-methoxy-2-(2-methoxyethoxy)ethane (10 mL), NaBH$_4$ (30 mg, 0.79 mmol) and LiCl (36 mg, 0.85 mmol) were added. The reaction mixture was refluxed for 2 hours and allowed to reach room temperature and quenched with water (5 mL). The solvent was evaporated under reduced pressure, the aqueous layer was extracted with CH$_2$Cl$_2$ (3 x 10 mL). The organic layer was collected, dried over anhydrous MgSO$_4$ and evaporated in vacuo to dryness. Column chromatography (EtOAc/NEt$_3$, 1:0.01) gave **3a**; yield: 190 mg (55%) as a colorless glass. ^{1}H NMR (250.13 MHz, CDCl$_3$): δ 1.00-1.90 (m, 10H, 5 x CH$_2$), 2.10 (bs, 1H, OH), 2.48-2.70 (m, 6H, N(CH$_2$)$_3$), 2.80-3.10 (m, 4H, N(CH$_2$)$_2$), 3.50 (s, 2H, CH$_2$OH), 3.70-4.00 (m, 5H, NCH$_2$ and CH$_3$), 6.75-7.00 (m, 4H, 4 x

CH), 7.10-7.30 (m, 2H, 2 x CH), 7.70 (dt, J = 7.6 Hz, 1H, CH), 8.54 (d, J = 4.9 Hz, 1H, CH).

4-(N-(2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)carbamoyl) bicyclo[2.2.1]heptan-1-yl)methyl 4-methylbenzenesulfonate (4a).

In a flame dried flask under argon atmosphere, compound **3a** (85 mg, 0.18 mmol) was dissolved in a mixture of pyridine (2 mL) and CH_2Cl_2 (10 mL), 4-methylbenzene-1-sulfonyl chloride (100 mg, 0.52 mmol) was added. This reaction mixture was stirred for 48 minutes at room temperature. The solvents were evaporated, the residue was dissolved in water and extracted with CH_2Cl_2 (3 x 5 mL). The organic phase was collected, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. Column chromatography (EtOAc/n-hexane/NEt$_3$, 1:0.10:0.01) afforded **4a**; yield: 91 mg (82%) as a white solid. ^{1}H NMR (250.13 MHz, CDCl$_3$): δ 1.00-1.90 (m, 10H, 5 x CH$_2$), 2.35 (s, 3H, CH$_3$), 2.48-2.70 (m, 6H, N(CH$_2$)$_3$), 2.80-3.10 (m, 4H, N(CH$_2$)$_2$), 3.70-4.00 (m, 7H, CH$_2$, NCH$_2$ and CH$_3$), 6.75-7.00 (m, 4H, 4 x CH), 7.10-7.30 (m, 2H, 2 x CH), 7.36 (d, J = 8 Hz, 2H, 2 x CH), 7.70 (dt, J = 7.6 Hz, 1H, CH), 7.77 (d, J = 8 Hz, 2H, 2 x CH), 8.54 (d, J = 4.9 Hz, 1H, CH). ^{13}C NMR (62.90 MHz, CDCl$_3$): δ 21.47, 32.08, 34.01, 45.27, 45.75, 46.99, 50.41, 53.23, 55.16, 55.53, 111.01, 117.89, 120.74, 122.65, 122.89, 123.47, 127.63, 129.64, 132.69, 137.98, 141.09, 144.50, 148.93, 152.03, 155.42, 174.71. HRMS (EI) m/z calcd for $C_{34}H_{42}N_4O_5S$, 618.2876; found, 619.2879 (M + H).

Methyl4-(N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)carbamo yl)cubane-1-carboxylate (2b).

In a flame dried flask under argon atmosphere, 4-methoxycarbonylcubane carboxylic acid (**1b**) (300 mg, 1.45 mmol) was dissolved in MeCN (20 mL) and thionyl chloride (145 μL) was added. The reaction mixture was refluxed at 70 °C for 45 minutes, the excess of thionyl chloride was totally evaporated under reduced pressure and coevaporated with MeCN (3 x 3 mL). To the corresponding acid chloride, WAY-100634 (455 mg, 1.45 mmol) and NEt$_3$ (224 μL) in MeCN (15 mL) were added and stirred at room temperature overnight. The solvent was evaporated, the residue was dissolved in

water and extracted with CH_2Cl_2 (3 x 15 mL). The organic phase was collected, dried over anhydrous Na_2SO_4 and evaporated to dryness. Column chromatography (EtOAc/NEt$_3$, 1:0.01) gave **2b**; yield: 581 mg (80%) as a white solid. ^{1}H NMR (200.13 MHz, CDCl$_3$): δ 2.50-2.70 (m, 6H, N(CH$_2$)$_3$), 2.80-3.00 (m, 4H, N(CH$_2$)$_2$), 3.60 (s, 3H, CH$_3$), 3.75 (s, 6H, 6 x CH), 3.90-4.05 (m, 5H, NCH$_2$ and CH$_3$), 6.70-7.05 (m, 4H, 4 x CH), 7.10-7.30 (m, 2H, 2 x CH), 7.60 (dt, J = 7.6 Hz, 1H, CH), 8.45 (d, J = 4.9 Hz, 1H, CH).

4-(Hydroxymethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl) cubane-1-carboxamide (3b).

In a flame dried flask under argon atmosphere, compound **2b** (393 mg, 0.786 mmol) was dissolved in (1-methoxy-2-(2-methoxyethoxy) ethane (20 mL), NaBH$_4$ (160 mg, 4.28 mmol) and LiCl (180 mg, 4.26 mmol) were added. The reaction mixture was refluxed overnight. After, the reaction mixture was quenched with water (15 mL), the solvent was evaporated under reduced pressure, the aqueous layer was extracted with CH_2Cl_2 (3 x 10 mL). The organic layer was collected, dried over anhydrous MgSO$_4$ and evaporated in vacuo to dryness. Column chromatography (EtOAc/NEt$_3$, 1:0.01) gave 186 mg of **3b** (50%) as a colorless glass. ^{1}H NMR (200.13 MHz, CDCl$_3$): δ 2.50-2.70 (m, 6H, N(CH$_2$)$_3$), 2.90-3.00 (m, 4H, N(CH$_2$)$_2$), 3.50-3.75 (m, 9H, 6 x CH and CH$_3$), 3.80 (s, 2H, CH$_2$OH), 3.90-4.10 (m, 2H, NCH$_2$), 5.00 (bs, 1H, OH), 6.70-7.00 (m, 4H, 4 x CH), 7.05-7.25 (m, 2H, 2 x CH), 7.70 (dt, J = 7.6 Hz, 1H, CH), 8.54 (d, J = 4.9 Hz, 1H, CH).

4-(Bromomethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl) cubane-1-carboxamide (4b).

Compound **3b** (180 mg, 0.38 mmol) was dissolved in CH_2Cl_2 (24 mL) and cooled to 0 °C, triphenyl phosphine (189 mg, 0.72 mmol) was added and followed by a dropwise addition of CBr$_4$ (142 mg, 0.43 mmol). The reaction mixture was stirred at 0 °C for 4 hours. The solvent was evaporated under reduced pressure at 0 °C. The residue was dissolved in eluent and purified with column chromatography (EtOAc/NEt$_3$, 1:0.01) to give 100 mg (49%) of **4b** as a colorless glass. δ 2.50-2.80 (m, 6H, N(CH$_2$)$_3$), 2.90-3.10 (m, 4H, N(CH$_2$)$_2$), 3.50-3.90 (m, 11H, 6 x CH, CH$_3$ and CH$_2$Br), 4.00-4.20 (m, 2H, NCH$_2$), 6.80-7.10 (m, 4H, 4 x CH), 7.10-7.30 (m, 2H, 2 x CH), 7.65 (dt, J = 7.6 Hz, 1H,

CH), 8.45 (d, J = 4.9 Hz, 1H, CH). ^{13}C NMR (50.32 MHz, CDCl$_3$): δ 35.93, 42.10, 44.70, 45.05, 45.31, 46.22, 50.38, 53.19, 55.16, 56.12, 56.02, 110.96, 117.92, 119.95, 120.75, 121.65, 122.71, 138.02, 140.92, 148.85, 153.05, 157.15, 171.84. HRMS (EI) m/z calcd for C$_{28}$H$_{31}$BrN$_4$O$_2$, 534.1630; found, 535.1649 (Br = 79, M + H) and 537.1635 (Br = 81, M + H).

Methyl4-(N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)carbamoyl) bicyclo[2.2.2]octane-1-carboxylate (2c).

In a flame dried flask under argon atmosphere, 4-(methoxycarbonyl) bicyclo[2.2.2]octane-1-carboxylic acid (**1c**) (250 mg, 1.18 mmol) was dissolved in MeCN (15 mL) and thionyl chloride (120 μL) was added. The reaction mixture was refluxed at 70 ^{o}C for 45 minutes, the excess of thionyl chloride was totally evaporated under reduced pressure and coevaporated with MeCN (3 x 3 mL). To the corresponding acid chloride, WAY-100634 (367 mg, 1.18 mmol) and NEt$_3$ (200 μL) in MeCN (10 mL) were added and stirred at room temperature overnight. The solvent was evaporated, the residue was dissolved in water and extracted with CH$_2$Cl$_2$ (3 x 15 mL). The organic phase was collected, dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness. Column chromatography (EtOAc/NEt$_3$, 1:0.01) gave **2c**; yield: 419 mg (70%) as a white solid. ^{1}H NMR (200.13 MHz, CDCl$_3$): δ 1.36-1.61 (m, 6H, 3 x CH$_2$), 1.72-1.90 (m, 6H, 3 x CH$_2$) 2.52-2.75 (m, 6H, N(CH$_2$)$_3$), 2.83-3.00 (m, 4H, N(CH$_2$)$_2$), 3.60 (s, 3H, CH$_3$), 3.88-4.05 (m, 5H, NCH$_2$ and CH$_3$), 6.70-7.05 (m, 4H, 4 x CH), 7.10-7.30 (m, 2H, 2 x CH), 7.60 (dt, J = 7.6 Hz, 1H, CH), 8.45 (d, J = 4.9 Hz, 1H, CH).

Methyl 4-(hydroxymethyl)bicyclo[2.2.2]octane-1-carboxylate (6).

In flame dried three-necked flask under argon atmosphere, 4-(methoxycarbonyl)bicyclo[2.2.2]octane-1-carboxylic acid (**1c**) (1.18 g, 5.23 mmol) was dissolved in freshly distilled THF (50 mL). This solution was cooled to 0 ^{o}C and a solution of 2 M borane dimethyl sulfide in THF (4.2 mL) was added dropwise. This reaction mixture was stirred at this temperature for 20 minutes then further stirred for 2.5 hours at room temperature, quenched with water (10 mL) and stirred for 2 hours at room temperature. The solvent was evaporated under reduced pressure and the residue was extracted with

EtOAc (3 x 15 mL). The organic layer was washed with unsaturated NaHCO$_3$ (2 x 10 mL), collected, dried over anhydrous MgSO$_4$ and evaporated in vacuo to dryness to give 1.061 g (96%) of **6** as a colorless oil. ^{1}H NMR (250.13 MHz, CDCl$_3$): δ 1.36-1.51 (m, 6H, 3 x CH$_2$), 1.72-1.86 (m, 6H, 3 x CH$_2$), 3.28 (s, 2H, CH$_2$OH), 3.50 (s, 3H, CH$_3$).

4-(Methoxycarbonyl)bicyclo[2.2.2]octan-1-yl)methyl 4-methylbenzenesulfonate (7).

In a flame dried flask under argon atmosphere, the appropriate alcohol **6** (2.11 g, 10.01 mmol) was dissolved in pyridine (15 mL) and 4-methylbenzene-1-sulfonyl chloride (1.91 g, 10.01 mmol) was added. This reaction mixture was stirred at room temperature overnight, the solvent was evaporated and the residue was dissolved in water and extracted with EtOAc (3 x 20 mL). The organic phase was separated, washed with unsaturated NaHCO$_3$ (2 x 10 mL), collected, dried over anhydrous Na$_2$SO$_4$ and evaporated under reduced pressure to dryness to give 3.60 g (98.5%) of **7** as white crystals. ^{1}H NMR (250.13 MHz, CDCl$_3$): δ 1.35-1.46 (m, 6H, 3 x CH$_2$), 1.68-1.84 (m, 6H, 3 x CH$_2$), 2.45 (s, 3H, CH$_3$), 3.50 (s, 3H, CH$_3$), 4.07 (q, J = 7 Hz, 2H, CH$_2$OTs), 7.34 (d, J = 8 Hz, 2H, 2 x CH), 7.77 (d, J = 8 Hz, 2H, 2 x CH).

Methyl 4-(iodomethyl)bicyclo[2.2.2]octane-1-carboxylate (8).

In closed vial, compound **7** (500 mg, 1.37 mmol) was dissolved in 2-methoxyethanol, also known as 'methylcellosolve' (25 mL), an excess of NaI (2.46 g, 16.42 mmol) was added and the reaction mixture was heated to 130 ^{o}C overnight. The solution was allowed to reach room temperature and the solvent was evaporated under reduced pressure. The residue was dissolved in water (10 mL), extracted with ether (3 x 10 mL). The organic layer was collected, dried (Na$_2$SO$_4$), filtered and evaporated to dryness to give 300 mg (68%) of **8** as a white solid after column chromatography (n-hexane/EtOAc, 1:0.04). ^{1}H NMR (250.13 MHz, CDCl$_3$): δ 1.43-1.55 (m, 6H, 3 x CH$_2$), 1.74-1.85 (m, 6H, 3 x CH$_2$), 3.05 (s, 2H, CH$_2$I), 3.50 (s, 3H, CH$_3$).

4-(Iodomethyl)bicyclo[2.2.2]octane-1-carboxylic acid (9).

Compound **8** (150 mg, 0.49 mmol) was dissolved in MeOH (10 mL), added to a solution of NaOH (19.5 mg, 0.49 mmol) in water (1 mL) and stirred at room temperature

overnight. Then, the solvent was evaporated under reduced pressure. The residue was dissolved in water and extracted with diethyl ether (3 x 5 mL). The organic phase was collected to get rid of any non-reacting starting material. The aqueous phase was collected and subsequently acidified with 6 N HCl to reach a pH 1, which was then extracted with EtOAc (3 x 5 mL), the organic phase was collected, dried (Na_2SO_4) and evaporated to dryness giving 74 mg (51%) of **9** as a white solid after column chromatography (n-hexane/EtOAc, 6:1). ^{1}H NMR (250.13 MHz, CDCl$_3$): δ 1.43-1.74 (m, 6H, 3 x CH$_2$), 1.80-1.85 (m, 6H, 3 x CH$_2$), 3.10 (s, 2H, CH$_2$I).

4-(Iodomethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)bicyclo[2.2.2]octane-1-carboxamide (4c).

In a dried closed reaction vial and under argon atmosphere, the reaction mixture of **9** (35 mg, 0.12 mmol) and dichloro(methoxy)methane (10.6 μL, 0.12 mmol) in CH$_2$Cl$_2$ (4 mL) was heated for four days at 60 ^{o}C. The volatile products (HCl and methyl formate) were removed. Then WAY-100634 (37.17 mg, 0.12 mmol) in CH$_2$Cl$_2$ (1 mL) and NEt$_3$ (24.1 mg, 0.24 mmol) were added to the corresponding acid chloride. The new reaction mixture was stirred for 1 hour at room temperature. The solvent was evaporated and the residue was dissolved in water (3 mL) and extracted with EtOAc (2 x 10 mL). The organic phase was collected, dried over anhydrous Na$_2$SO$_4$ and solvent was evaporated under reduced pressure to give 44 mg (63%) of **4c** as a colorless glass after column chromatography (CH$_2$Cl$_2$/MeOH, 20:1). ^{1}H NMR (250.13 MHz, CDCl$_3$): δ 1.25-1.45 (m, 6H, 3 x CH$_2$), 1.60-1.80 (m, 6H, 3 x CH$_2$), 2.55-2.80 (m, 6H, N(CH$_2$)$_3$), 2.95 (s, 2H, CH$_2$I), 3.00-3.15 (m, 4H, N(CH$_2$)$_2$), 3.80-4.00 (m, 5H, CH$_3$ and NCH$_2$), 6.80-7.05 (m, 4H, 4 x CH), 7.22-7.33 (m, 2H, 2 x CH), 7.74 (dt, J = 7.6 Hz, 1H, CH), 8.51 (d, J = 4.9 Hz, 1H, CH). ^{13}CNMR (100.62 MHz, CDCl$_3$): δ 22.90, 29.32, 30.72, 31.38, 42.36, 48.86, 50.56, 53.48, 55.37, 55.71, 111.34, 118.12, 120.98, 122.68, 122.82, 123.34, 138.21, 141.37, 149.01, 152.28, 156.84, 177.76. HRMS (EI) m/z calcd for C$_{28}$H$_{37}$IN$_4$O$_2$, 588.1961; found, 589.1972 (M + H).

Radiotracers

Radiofluorination:

[^{18}F]F$^-$ was produced by the ^{18}O(p,n)^{18}F nuclear reaction with an IBA 18/9 cyclotron. After irradiation, [^{18}F]F$^-$ was trapped on a Waters QMA SepPak column (carbonate form). It was eluted from the anion exchange column with 1 ml of acetonitrile:water (9:1 v:v) containing Kryptofix[2.2.2] and potassium carbonate. The solution was dried under helium flow and reduced pressure at 90 °C for 15 minutes. To remove residual water, 0.5 ml of acetonitrile was added and the solution was dried again. At room temperature, the precursor (**4a**, **4b** or **4c**) in 0.5 ml of acetonitrile was added. Test reactions were performed with small amounts of activity (0.5-1 GBq) to find conditions for which the labelling yield was at least 50%. All reactions were carried out utilizing homemade, remotely controlled equipment.

The yields were monitored by radio-thin-layer chromatography (radio-TLC) using Merck precoated silica gel F-254 plates (thickness 0.3 mm) and dichloromethane/methanol (95:5 v:v) as eluent, and by HPLC (kromasil C18-10 μm column, 4.6 mm x 250 mm; eluent (MeOH/H$_2$O/DIPA, 80:20:0.02). Radio thin-layer chromatography (radio-TLC) plates were exposed for 5 minutes to a Molecular Dynamics phosphor imager plate and subsequently scanned with a Storm 820 imager (Molecular Dynamics). Obtained images were analyzed with ImageQuant 5.2. Analytical HPLC was performed with a Jasco PU1580 pump, an in-line Jasco UV2075 UV detector (wavelength 254 nm), a flow through NaI (Tl) crystal scintillation detection system and Raytest Gina-NT for data acquisition.

For determining the specific activity (SA) at the end of synthesis (EOS), the UV absorbance on HPLC was calibrated using a 50 μl injection of the cold compounds. For all three compounds a linear correlation (R^2 >0.995) was obtained in a concentration range of 0.5-10 nmol/mL.

[^{18}F]4-(Fluoromethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)bicyclo[2.2.1]heptane-1-carboxamide ([^{18}F]5a).

Good results for the labelling were found when 4 mg (6.5 μmol) of precursor **4a** in dry MeCN (500 μL) was added to the dried radiofluoride, 2,2,2-kryptofix (13 mg) and

K_2CO_3 (2 mg), and heated at 130 °C for 30 minutes in a closed vial. Under these conditions the reaction was performed twice, first for the biodistribution study and a second time for the PET study with ^{18}F amounts of 26 c.q. 24 GBq. After the addition of water (0.5 mL), the solution was injected on HPLC using a C18-10 μm column (kromasil, 4.6 mm x 250 mm) and (MeOH/H_2O/DIPA, 60:40:0.02) as eluent with a flow of 1 mL/min (t_R = 24-28 minutes). The collected radioactive fraction (4 mL) was diluted with water (30 mL) and the product was trapped on a SepPak C18 plus cartridge. The SepPak was rinsed with water (20 mL) and inversely eluted with EtOH (1 mL). The ethanolic fraction was further formulated with 9 mL of citrate buffer (pH 4.8) to give [^{18}F]**5a** in a concentration of 496 c.q. 433 MBq/mL. The total synthesis time was about 105 minutes. So, the decay corrected overall yields were 37 c.q. 35%. Analytical HPLC (kromasil C18-10 μm column, 4.6 mm x 250 mm); eluent (MeOH/H_2O/DIPA, 65:35:0.02), of a 50 μL sample revealed a radiochemical purity of [^{18}F]**5a** of 99.1 c.q. 99.8% and a SA at EOS of 132 c.q. 76 GBq/μmol. The UV channel showed besides **5a** (t_R = 17.2 minutes, 75 c.q. 55%) some other detectable signals at t_R = 13.8 minutes (15 c.q. 20%), t_R = 14.5 minutes (0 c.q. 15%) and t_R = 16.4 minutes (10 c.q. 10%).

[^{18}F]4-(Fluoromethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)cubane-1-carboxamide ([^{18}F]5b).

Formation of [^{18}F]**5b** already proceeded smoothly when 2 mg (3.7 μmol) of precursor **4b** in dry MeCN (500 μL) was added to the dried radiofluoride, 2,2,2-kryptofix (13 mg) and K_2CO_3 (2 mg), and heated at 110 °C for 15 minutes in a closed vial. ^{18}F amounts used for both the biodistribution and the PET study were 22 GBq. After the addition of water (0.5 mL) the solution was injected on HPLC using a C18-10 μm column (kromasil, 4.6 mm x 250 mm); eluent (MeOH/H_2O/DIPA, 60:40:0.02) with a flow of 1 mL/min and product was collected at (t_R = 23-27 minutes). The radioactive fraction (4 mL) was diluted with water (30 mL) and the product was trapped on a SepPak tC18 plus cartridge. The SepPak was rinsed with water (20 mL) and inversely eluted with EtOH (1 mL). The ethanolic fraction was further formulated with 9 mL of citrate buffer (pH 5.0) to give [^{18}F]**5b** in a concentration of 299 c.q. 474 MBq/mL. With a total synthesis time of about 90 minutes, the decay corrected yield was 24 c.q. 38%. Analytical HPLC (kromasil

C18-10 μm column, 4.6 mm x 250 mm); eluent (MeOH/H$_2$O/DIPA, 65:35:0.02), of a 50 μL sample revealed a radiochemical purity of [^{18}F]**5b** of 98.1 c.q. 98.6% and a SA at EOS of 119 c.q. 138 GBq/μmol. The UV channel showed besides **5b** (t_R = 16.9 minutes, 45 c.q. 50%) two other detectable signals at t_R = 13.8 minutes (30 c.q. 15%) and t_R = 15.9 minutes (25 c.q. 35%).

[^{18}F]4-(Fluoromethyl)-N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)bicyclo[2.2.2]octane-1-carboxamide ([^{18}F]5c).

For the formation of [^{18}F]**5c** the reaction temperature had to be raised. The amounts of 2,2,2-kryptofix and K$_2$CO$_3$ were reduced to prevent decomposition of the starting material. Best results were found when 4 mg (6.8 μmol) of precursor **4c** in dry MeCN (500 μL) was added to the dried radiofluoride, 2,2,2-kryptofix (3.25 mg) and K$_2$CO$_3$ (0.5 mg), and heated at 150 ^{o}C for 15 minutes in a closed vial. The ^{18}F amounts used for the biodistribution study and for the PET study was 24 c.q. 29 GBq. After the addition of water (0.5 mL) the solution was injected on HPLC using a C18-10 μm column (kromasil, 4.6 mm x 250 mm) and (MeOH/H$_2$O/DIPA, 65:35:0.02) as eluent with a flow of 1 mL/min (t_R = 22-26 minutes). The collected radioactive fraction (4 mL) was diluted with water (30 mL) and the product was trapped on a SepPak C18 plus cartridge. The SepPak was rinsed with water (20 mL) and inversely eluted with EtOH (1 mL). The ethanolic fraction was further formulated with 9 mL of citrate buffer (pH 4.8) to give [^{18}F]**5c** in a concentration of 531 c.q. 740 MBq/mL. The total synthesis time was 90 minutes and the decay corrected yield 39 c.q. 45%. Analytical HPLC (kromasil C18-10 μm column, 4.6 mm x 250 mm); eluent (MeOH/H$_2$O/DIPA, 70:30:0.02), of a 50 μL sample revealed a radiochemical purity of [^{18}F]**5c** of 99.8 for both productions and a SA at EOS of 90 c.q. 120 GBq/μmol. The UV channel showed besides **5c** (t_R = 16.0 minutes, 80 c.q. 80%) some other signals at t_R = 12.6 minutes (0 c.q. 20%), t_R = 14.2 minutes (15 c.q. 0%) and t_R = 19.4 minutes (5 c.q. 0%).

Biodistribution studies

Animal experiments were performed with Wistar rats (male, body weight around 230 g, Harlan, Horst, The Netherlands). Approval of the institutional animal ethics

committee was obtained and all experiments were carried out in accordance with both the Dutch Law on Animal Experimentation and guidelines of the institutional committee on animal experimentation. Rats (four per study) were anesthetized with 2% isoflurane, 0.5 oxygen L/min and received an intravenous injection of 200 μL of the sterilized radiolabeled compound via the tail vein. Rats were sacrificed at 45 minutes after injection and tissues of interest were removed, weighed and measured for radioactivity using a LKB Wallac 1282 CompuGamma CS.

For calculation of the injected dose, five aliquots of the injected solution were weighed and counted for radioactivity. Results were decay corrected and expressed as percentage of injected dose per gram of tissue $\pm$ standard deviation (%ID/g $\pm$ S.D.). Data were analyzed by ANOVA (one-way analysis of variance) followed by Dunnett's test to correct for multiple comparison. $P \leq 0.05$ indicate significant difference.

PET studies

Additional *in vivo* PET studies of [^{18}F]**5a**, [^{18}F]**5b** and [^{18}F]**5c** were performed using a double LSO/LYSO layer High Resolution Research Tomograph (HRRT; CTI/Siemens, Knoxville, TN, USA). Performance characteristics of this scanner have been reported previously.[37] The combination of high spatial resolution and high sensitivity makes the HRRT an ideal PET scanner for dynamic studies in small laboratory animals. First, for attenuation and scatter correction, a transmission scan was acquired using a 740 MBq 2-dimensional (2D) fan-collimated ^{137}Cs (662 keV) moving point source (9). Next, a dynamic emission scan of 60 minutes was acquired following administration of the radiotracer. Data were acquired in list mode and rebinned into the final frame sequence: 6 x 10, 1 x 20, 2 x 30, 2 x 60 seconds, 2 x 2.5, 2 x 5 and 4 x 10 minutes. Following corrections for decay, dead time, scatter and randoms, scans were reconstructed using an iterative 3D ordered-subsets weighted least-squares (3D-OSWLS) method.

Prior to the PET scan, rats (n = 2 for each study group) were anesthetized (isofluran 2%, oxygen 0.45 volume %) and cannulated via the jugular vein for venous access. After cannulation, rats were positioned in the PET scanner (2 rats placed side by side on the camera bed and kept under anesthesia breathing through a ventilation mask

(Isofluran 1-2%, oxygen 0.45 volume %) and body temperature was kept within normal range (37-38 °C)).

Rats were injected through the cannula with sterile 0.9% NaCl or WAY-100635 (2 mg/kg) 5 minutes prior to the radioligand (20 μL) injection. Rats were also sacrificed and tissues dissected immediately after the PET study. Both studies were done with the same batch of the radioligand with a time interval of 150 minutes; the blocking study the last.

Time activity curves of the radiotracer, reflecting delivery of the tracer to the brain, were derived for predefined volumes of interest (VOIs), in rats, the VOIs were drawn in ellipsoids over the whole brain. The position of all VOI was defined visually on a summation image of time frames from 0.5-60 minutes. Next, this VOI was projected onto all dynamic frames, resulting in a brain time-activity curve of the tracer, using the software package AMIDE (Amide 0.9.1 http://amide.sourceforge.net). The standardized uptake value (SUV = radioactivity per cm^3 / injected radioactivity per gram of bodyweight) was calculated based on the VOI activity, injected activity dose and animal weight.

References

1. Passchier J, van Waarde A. Visualisation of serotonin-1A (5-HT$_{1A}$) receptors in the central nervous system. *Eur J Nucl Med* 28 113-129; 2001.

2. Handley SL, McBlane JW. 5-HT drugs in animal models of anxiety. *Psychopharmacology* 112 13-20; 1993.

3. Drevets WC, Frank E, Price CJ, Kupfer DJ, Holt D, Greer PJ, Huang Y, Gautier C, Mathis C. PET Imaging of serotonin 1A receptor binding in depression. *Biol Psychiatry* 46 1375-1387; 1999.

4. Matsubara S, Arora RC, Meltzer HY. Serotonergic measures in suicide brain -5-HT$_{1A}$ binding-sites in frontal cortex of suicide victims. *J Neural Transmissions* 85 181-194; 1991.

5. Kepe V, Barrio JR, Cheng Huang S, Ercoli L, Siddarth P, Shoghi Jadid K, Cole GM, Satyamurthy N, Cummings JL, Small GW, Phelps ME. Serotonin 1A receptors in the living brain of Alzheimer's disease patients. *PNAS* 103 702-707; 2006.

6. Pike VW, Halldin C, Wikström HV. Radioligands for the study of the brain 5-HT$_{1A}$ receptors *in vivo*. *Prog Med Chem* 38 189-247; 2001.

7. Bantick RA, Deakin JFW, Grasby P M. The 5-HT$_{1A}$ receptor in schizophrenia: a promising target for novel atypical neuroleptics? *Psychopharmacology* 15 37-46; 2001.

8. Millan MJ. Improving the treatment of schizophrenia: Focus on serotonin (5-HT)$_{1A}$ receptors. *J Pharmacol Exp Ther* 295 853-861; 2000.

9. Yasuno F, Suhara T, Ichimiya T, Takano A, Ando T, Okubo Y. Decreased 5-HT$_{1A}$ receptor binding in amygdala of schizophrenia. *Biol Psychiatry* 55 439-444; 2004.

10. Fletcher A, Bill DJ, Corn IA, Forster EA, Jones D, Reilly Y. A pharmacological profile of WAY-100635, a potent and selective 5-HT$_{1A}$ receptor antagonist. *Br J Pharmacol* 112:91P; 1994.

11. Cliffe IA. A retrospect on the discovery of WAY 100635 and the prospect for improved 5-HT$_{1A}$ receptor PET radioligands. *Nucl Med Biol* 27 441-447; 2000.

12. Pike VW, McCarron JA, Hume SP, Ashworth S, Opacka-Juffry J, Osman S, Lammertsma AA, Poole KG, Fletcher A, White AC, Cliffe IA. Pre-clinical development of a Radioligand for studies of central 5-HT-$_{1A}$ receptors *in vivo* [^{11}C]- WAY 100635. *Med Chem Res* 5 208-227; 1994.

13. Pike VW, Halldin C, Wikström H, Marchais S, McCarron JA, Sandell J, Nowicki B, Swahn C-G, Osman S, Hume SP, Constantinou M, Andree B, Farde L. Radioligands for the study of brain 5-HT1A receptors *in vivo*–Development of some new analogues of WAY. *Nucl Med Biol* 27 449-455; 2000.

14. Krasikova RN, Andersson J, Truong P, Nag S, Shchukin EV, Halldin C. A fully automated one-pot synthesis of [carbonyl-^{11}C]WAY-100635 for clinical PET applications. *Appl Radiat Isot* 67 73-78; 2009.

15. Passchier J, Elsinga PH. Quantitative imaging of 5-HT1A receptor binding in healthy volunteers with [^{18}F]MPPF. *Nucl Med Biol* 27 473-476; 2000.

16. Lang L, Jagoda E, Schmall B, Vuong BK, Richard Adams H, Nelson DL, Carson RE, Eckelman WC. Development of fluorine-18- labeled 5-HT$_{1A}$ antagonist. *J Med Chem* 42 1576-1582; 1999.

17. Passchier J, van Waarde A, Doze P, Elsinga PH, Vaalburg W. Influence of P-glycoprotein on brain uptake of [^{18}F]MPPF in rats. *Eur J Pharmacol* 407, 273-280; 2000.

18. Raub TJ. P-glycoprotein recognition of substrates and circumvention through rational drug design. *Mol Pharm* 3 3-25; 2006.

19. Lang L, Jagoda E, Ma Y, Sassaman MB, Eckelman WC. Synthesis and *in vivo* biodistribution of [^{18}F] labeled 3-cis-3-trans,4-cis-, and 4-trans-fluorocyclohexane derivatives of WAY 100635. *Bioorg Med Chem* 14 3737-3748; 2006.

20. Vuong BK, Kiesewetter DO, Lang L, Ma Y, Eckelman WC, Channing MA. An automated one-step one-pot [^{18}F]FCWAY synthesis: development and minimization of chemical impurities. *Nucl Med Biol* 34 433-438; 2007.

21. Saigal N, Pichik R, Easwaramoorthy B, Collins D, Christian BT, Shi B, Narayanan TK, Potkin SG, Mukherjee J. Synthesis and biological evaluation of novel serotonin 5-HT$_{1A}$ receptor radioligand, ^{18}F-labeled Mefway, in rodents and imaging by PET in nonhuman primate, *J Nucl Med* 47 1697-1706; 2006.

22. Al Hussainy R, Verbeek J, van der Born D, Booij J, Herscheid JDM. Design, synthesis and *in vitro* evaluation of bridgehead fluoromethyl analogs of *N*-{2-[4-(2-methoxyphenyl)piperazin-1-yl}-*N*-(pyridine-2-yl)cyclohexanecarboxamide (WAY-100635) for the 5-HT$_{1A}$ receptor. *Eur J Med Chem* 46 5728-5735; 2011.

23. Al Hussainy R, Verbeek J, van der Born D, Braker AH, Leysen JE, Knol RJ, Booij J, Herscheid JDM. Design, synthesis, radiolabeling, and *in vitro* and *in vivo* evaluation of bridgehead Iodinated analogues of N-{2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl}-N-(pyridin-2-yl)cyclohexanecarbox amide (WAY-100635) as potential SPECT ligands for the 5-HT$_{1A}$ receptor. *J Med Chem* 54 3480-3491; 2011.

24. Ottenheijm HCJ, Tijhuis WM. Acid chlorides from α- keto acids with α,α- dichloromethyl methyl ether: pyruvoyl chloride. *Org Syn Coll* 7 467; 1990 and 61 1; 1983.

25. Carson RE, Wu Y, Lang L, Ma Y, Der MG, Herscovitch P, Eckelman WC. Brain uptake of the acid metabolites of F-18- labeled WAY 100635 analogs. *J Cereb Blood Flow Metab* 23 249-260; 2003.

26. Hoon Ryu Y, Liow J, Zoghbi S, Fujita M, Collins J, Tipre D, Sangare J, Hong J, Pike VW, Innis RB. Disulfiram inhibits defluorination of ^{18}F-FCWAY, reduces bone radioactivity, and enhances visualization of radioligand binding to serotonin 5-HT$_{1A}$ receptors in human brain. *J Nucl Med* 48 1154-1161; 2007.

27. Pike VW, PET radiotracers: crossing the blood-brain barrier and surviving metabolism. *Trends Pharmacol Sci* 30 431-440; 2009.

28. Maksić ZB, Eckert- Maksić M, Randić M. Correlation between C-H and C-C spin-spin coupling constants and *s* character of hybrids calculated by the maximum overlap method. *Theor Chim Acta (Berl)* 22 70-79; 1971.

29. Randić M, Stefanović D. Hybridisation in norbornane and some analogues by the method of maximum overlap. *J Chem Soc (B)* 423-425; 1968.

30. Choi SY, Eaton PE, Hollenberg PF, Liu KE, Lippard SJ, Newcomb M, Putt DA, Upadhyaya SP, Xiong Y. Regiochemical variations in reactions of methylcubane with *tert*-butoxyl radical, cytochrome P-450 enzymes, and a methane monooxygenase system. *J Am Chem Soc* 118 6547-6555; 1996.

31. Newcomb M, Shen R, Choi SY, Toy PE, Hollenberg PF, Vaz AND, Coon MJ. Cytochrome P450-catalyzed hydroxylation of mechanistic probes that distinguish between radicals and cations. Evidence of cationic but not for radical intermediates. *J Am Chem Soc* 122 2677-2686; 2000.

32. Plenevaux A, Weissmann D, Aerts J, Lemaire C, Brihaye C, Degueldre C, Le Bars D, Comar D, Pujol JF, Luxen A. Tissue distribution, autoradiography, and metabolism of 4-(2'-Methoxyphenyl)-1-[2'-[*N*-(2"-Pyridinyl)-*p*-[^{18}F]Fluorobenzamido]ethyl]piperazine (*p*-[^{18}F]MPPF), a new serotonin 5-HT$_{1A}$ antagonist for positron emission tomography: An *in vivo* study in rats. *J Neurochem* 75 803-811; 2000.

33. Chemel BR, Roth BL, Armbruster B, Watts VJ, Nichols DE. WAY-100635 is a potent dopamine D$_4$ receptor agonist. *Psychopharmacology* 188 244-251; 2006.

34. Park BK, Kitterinham NR, M O'Neill P. Metabolism of fluorine-containing drugs. *Annu Rev Pharmacol Toxicol* 41 443-470; 2001.

35. Tipre DN, Zoghbi SS, Liow JS, Green MV, Seidel J, Ichise M, Innis RB, Pike VW. PET imaging of brain 5-HT$_{1A}$ receptors in rat *in vivo* with [^{18}F]FCWAY and improvement by successful inhibition of defluorination with miconazole. *J Nucl Med* 47 345-353; 2006.

36. Shetty HU, Zoghbi SS, Simeon FG, Liow JS, Brown AK, Kannan P, Innis RB, Pike VW. Radiodefluorination of 3-Fluoro-5-(2-(2-[^{18}F](fluoromethyl)-thiazol-4-yl)ethynyl)benzonitrile ([^{18}F]SP203), a radioligand for imaging brain metabotropic glutamate subtype-5 receptors with positron emission tomography, occurs by glutathionylation in rat brain. *J Pharmacol Exp Ther* 327 727-735; 2008.

37. De Jong HW, van Velden FH, Kloet RW, Buijs FL, Boellaard R, Lammertsma AA. Performance evaluation of the ECAT HRRT: an LSO-LYSO double layer high resolution, high sensitivity scanner. *Phys Med Biol* 52 1505-1526; 2007.