Synthesis and evaluation of novel serotonin 4 receptor radiotracers for single photon emission computed tomography

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Keywords: Molecular Imaging, radioligand, serotonin, 5-HT₄, SPECT

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Abstract

Despite its implication in several physiological and pathological processes the serotonin subtype-4 receptor (5-HT₄R) has seen limited effort for the development of radiolabeling agent especially concerning single photon emission computed tomography (SPECT). Bearing an ester function, the available ligands are rapidly susceptible to hydrolysis which limits their use *in vivo*. In this study the synthesis of iodinated benzamide and ketone analogs were described. Their affinity for the 5-HT₄R and their lipophilicity were evaluated and the most promising derivatives were evaluated *ex vivo* for their binding to the receptor and for their ability to displace the reference ligand [¹²⁵I]-SB207710.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) plays a central role in neurotransmission through its interaction with several receptor subtypes, and the deregulation of this system has been implicated in numerous pathologies [1]. In order to determine the implication of each receptor

subtypes in these diseases, intense research has been conducted during the last few years to develop selective radioligands of interest for positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging [2,3]. Among those receptors, 5-HT₄R are of particular interest. They have been described for the first time by Dumuis in 1988 and are positively linked to adenylate cyclase and could control the release of a wide variety of neurotransmitters [4]. These receptors are expressed in the central nervous system (CNS) and control brain physiological functions such as learning and memory, feeding and mood behaviour but they are also located in the periphery and are implicated in gastro-intestinal transit [5]. Interactions with peripheral 5-HT₄R appear beneficial in gastrointestinal disorders [6], while interactions with the central 5-HT₄R, mainly with agonists, result in cognitive improvement after chronic or acute administration [7]. For this reason it is now postulated that several major devastating illnesses could benefit from 5-HT₄R-directed therapy including Alzheimer's disease (AD) [8] or depression [9].

Over the years, potent and selective ligands have been developed towards the 5-HT₄R [10] which are mainly based on a typical 4-amino-5-chloro-2-methoxybenzoyl residue already described in the non-selective benzamide metoclopramide [11], as well as in the benzoate ML10302 and the benzophenone RS67,333 (Figure 1A) [12]. In order to specify *in vivo* the 5-HT₄R physiological or pathological role, selective radioligands have been developed for SPECT and PET such as benzodioxane [¹²³I]SB-207710 ([¹²³I]**1**) [13] or its chlorinated analogue [¹¹C] SB-207145 ([¹¹C]**2**) (Figure 1B) [14]. The latter remains the only radiotracer that has been evaluated in human studies [15]. Two radiotracers containing fluorine-18 [¹⁸F]MNI-698 ([¹⁸F]**3**) and [¹⁸F]MNI-699 ([¹⁸F]**4**) were described in the same chemical series but possessing a radioactive atom as a substituent of the piperidine alkyl chain [16]. Among them [¹⁸F]**3** gave promising results for imaging 5-HT₄R in the brain in monkey [17,18]. More recently two novel tracers [¹¹C]RX-1 ([¹¹C]**5**) and [¹⁸F]RX-2 ([¹⁸F]**6**) were developed and evaluated for PET imaging [19]. However each of these ligands containing an ester function are susceptible to ester hydrolysis *in vivo* which could limit their accuracy. Other chemical series were then explored with the recent development of azaphenanthrene derivatives [¹²⁵I]**7** and [¹²⁵I]**8** (Figure 1) [20,21]. The amide modulation could be of particular interest since this function is present in R-91150 which was developed as brain 5-HT_{2A}R SPECT radiotracers [23,24].

Figure 1. A: Structures of reference 5-HT₄R ligands; **B:** 5-HT₄R PET and SPECT radiotracers; **C:** 5-HT_{2A}R SPECT radiotracer; **D:** targeted structures.

2. Results

2.1. Chemistry

Despite the traditionally reported strategy for obtaining iodinated single photon emission computed tomography radiotracers, based on a iododestannylation reaction of a stannylated precursor [25], and while our structures appear to be favorable for this reaction, we focused on the introduction of iodine atoms on non-functionalized precursors using electrophilic aromatic substitution reactions.

Initially, we planned to synthesize iodinated derivatives without radioactive source, by generating I₂ from NaI using an oxidant in acidic conditions [26]. Obtaining the iodinated derivatives from the non-functionalized compounds allowed us to confirm the selective halogenation at the expected position, to evaluate their potential affinity toward 5-HT₄R and to validate the feasibility of this procedure for the radiolabeling of our non-functionalized derivatives. This strategy will also allow us to avoid the synthesis of stannylated precursor, typically used in this kind of radiolabeling strategies.

For the design of non-functionalized aryl ketone derivatives, acids **11a-b** were synthesized in a three-step reaction from 4-aminosalicylic acid: an esterification of carboxylic acid, an alkylation of phenol with an ethyl or a 2-(fluoro)ethyl chain and a saponification reaction (Scheme 1). Compounds **14a-b** were finally prepared according to the general route as previously described:

formation of β -keto ester, subsequent alkylation with a piperidine derivative, saponification-decarboxylation reaction, deprotection of *tert*-butoxycarbonyl group under acidic conditions and alkylation of the piperidine with (bromomethyl)cyclohexane (Scheme 1) [27].

Scheme 1. Synthetic route of aryl ketone derivatives **14a-b**^a

^aReagents, conditions and yields: (a) H_2SO_4 cc., MeOH, 16h, reflux, 90%; (b) iodoethane, K_2CO_3 , DMF, 70°C , overnight, 61%; (c) 2-fluoroethyl 4-methylbenzenesulfonate, K_2CO_3 , DMF, 110°C , 2h, 62%; (d) 1N NaOH aq., EtOH, rt, overnight, 94-96%; (e) CDI, dry THF, rt, 15h, then potassium 3-ethoxy-3-oxopropanoate, MgCl_2 , 40°C , 24h, 23-36%; (f) *tert*-butyl 4-(iodomethyl)piperidine-1-carboxylate, K_2CO_3 , DMF, rt, 48h; (g) KOH, EtOH/ H_2O (5:1), reflux, 5h, 70-74% over two steps; (h) TFA, DCM, rt, 1h; (i) bromomethylcyclohexane, K_2CO_3 , 110°C , 5h, 43-58% over two steps.

For the design of non-functionalized benzamide derivatives, aminopiperidine chains **16a-b** were synthesized in a two-step reaction, first by alkylation of the piperidine moiety with alkyl halides, then by reduction of amides using LiAlH_4 . Target compounds **17a-d** were finally obtained by a peptide coupling between carboxylic acids **11a-c** (previously synthesized, except commercially available 4-amino-2-methoxy-benzoic acid **11c**) and substituted aminopiperidines **16a-b** (Scheme 2).

Scheme 2. Synthetic route of benzamide derivatives **17a-d**^b

^bReagents, conditions and yields: (j) alkyl-halogenated derivatives, K₂CO₃, EtOH, reflux, 15-24h, 92-93%; (k) LiAlH₄, dry THF, addition 0°C then rt, 1-2h, reflux, 3h, 71-96%; (l) EDCI, HOBt, Et₃N, DMF, rt, 18-72h, 17-44%.

Iodinations from non-functionalized aryl ketone derivatives **14a-b** and benzamide derivatives **17a-d** were performed using NaI as the source of iodine in a mixture of acetic acid/hydrogen peroxide solution 30%. A total conversion was observed, after a purification by flash chromatography on silica gel column and compounds **18a-f** were obtained in 18-71% isolated yields (Scheme 3). Selective halogenation at the expected position validated the feasibility of our strategy.

Scheme 3. Synthesis of iodinated compounds **18a-f**^c and ¹²⁵I radiolabeling of compounds **17a-b**^c

^cReagents, conditions and yields: (m) NaI, acetic acid/30% H₂O₂ 2:1, rt, 1-2h, 18-71%; (n) Na¹²⁵I, acetic acid/30% H₂O₂ 2:1, rt, 30 min, 47-61%.

2.2 Synthesis of radioligand

The effectiveness of our synthetic route was based on a final electrophilic iodination step. Considering the *in vitro* activities of the iodinated compounds (Table 1), radioiodination of **17a-b** was performed (Scheme 3). [¹²⁵I]**18c-d** were obtained using Na¹²⁵I as the radioactive iodine source and peracetic acid (formed *in situ* by the reaction of H₂O₂ and acetic acid) as the oxidant in order to *in situ* generate iodine and to conduct the aromatic electrophilic substitution (Scheme 4). Radiotracers were isolated by a linear gradient HPLC run.

2.3 In vitro assays

According to the work described by Grossman *et al.*,^[28] precursors **14a-b**, **17a-d** and final derivatives **18a-f**, were evaluated *in vitro* for their potential guinea-pig 5-HT₄R affinity using a displacement assay of the tritiated ligand [³H]-GR113808, a specific and highly potent 5-HT₄R antagonist used to label specific binding sites in human or guinea-pig brain (Table 1).

Table 1. 5-HT₄R affinity for the compounds **14a-b**, **17a-d** and **18a-f**; calculated and measured lipophilicities and permeabilities in the PAMPA-BBB assay with their predictive penetration in the CNS for the compounds **18a-f**.

17c	NH	CH ₃		n.d. 72% / 40% ^g	n.d.	n.d.	n.d.	n.d.
18e	NH	CH ₃		13.9 ± 3.7 (n = 3) ^c 100% / 29%	3.59 - 5.25	5.0 ± 0.4	31.60	CNS +
17d	NH	CH ₃		n.d. 79% / 5% ^g	n.d.	n.d.	n.d.	n.d.
18f	NH	CH ₃		68.7 ± 11.4 (n=3) ^c 96% / 11%	2.36 - 3.84	3.5 ± 0.4	22.68	CNS +

n.d. not determined / ^a calculated with MarvinSketch / ^b calculated with Molinspiration / ^c guinea pig receptors / ^d human receptors

2.4 Imaging experiments

As detailed in the discussion section the most active *in vitro* compounds **18c-d** were selected for further preclinical evaluation as 5-HT₄R SPECT radiotracers. Following their radioiodination [¹²⁵I]**18c** and [¹²⁵I]**18d** were injected in the tail vein of rats and SPECT images were obtained (Figure 2). As illustrated by representative images [¹²⁵I]**18c** and [¹²⁵I]**18d** are able to enter the brain and are detected by the SPECT scanner. However, the brain distribution of both [¹²⁵I]**18c** and [¹²⁵I]**18d** did not show a specific accumulation in 5-HT₄R rich region, such as olfactory tubercles, caudate-putamen, in comparison with the cerebellum, a region devoid of 5-HT₄R according to *in vivo* SPECT imaging and *ex vivo* autoradiograms (Figures 2-3).

Figure 2. *In vivo* SPECT imaging showing activity distribution in the brain after injection of [^{125}I]18c or [^{125}I]18d. Representative MRI images in coronal (A), sagittal (B) and axial (C) planes. Arrows indicate areas of interest: olfactory tubercles (yellow), Caudate-putamen (white) and cerebellum (green). Images of SPECT scan between 10 and 60 minutes after injection of [^{125}I]18c (D-F) or [^{125}I]18d (G-I) coregistered with MRI template.

Figure 3. *Ex vivo* autoradiograms in the brain after injection of [125 I]**18c** or [125 I]**18d**. Anatomic atlas templates adapted from the Paxinos and Watson atlas (A), autoradiograms obtained 60 minutes after injection of [125 I]**18c** (B) or [125 I]**18d** (C). Cx: cortex, Ce: cerebellum, Hip: hippocampus, Ip: interpeduncular nucleus, Tu: olfactory tubercles, St: striatum, Thal: thalamus.

Being able to penetrate the brain and to accumulate in different regions of the brain, we decided to evaluate the capacity of our ligands to target the 5-HT₄R. A competition experiment was conducted between the specific 5-HT₄R radiotracer [125 I]**1** and our ligands **18c-d** and non radioactive **1** (Figure 4). In this *in vitro* study rat brain slides were first incubated with [125 I]**1** before being treated with increasing concentration of **18c** and **18d** (from 0.01 to 10 μ M, Figure 4, B-F) or 1 μ M of **1** (Figure 4, G). **18c-d** can inhibit the specific binding of [125 I]**1** on 5-HT₄R (Figure 4).

Figure 4. *In vitro* competition between [125 I]**1** and **18c**, **18d** or **1**. Localisation of olfactory tubercles (Tu) is shown in a coronal slice, adapted from the Paxinos and Watson atlas (A). Representative autoradiograms obtained by incubation of [125 I]**1** in the presence of growing concentrations (0, 0.01, 0.1, 1, 10 μ M, from B to F, respectively) of **18c** (topline) or **18d** (line below) or in the presence of 1 μ M of **1** (G).

3. Discussion

Our synthetic strategy led to the development of final derivatives **18a-f**, possessing an iodine atom on their structure which could be easily introduced through a late stage diversification process. Two of them were designed to possess a ketone function (**18a-b**) and four (**18c-f**) were obtained with an amide linker. Our initial objectives were to assess the influence of these pharmacomodulations on the biological activity but also on their lipophilicity and their ability to cross the BBB. All the synthesized derivatives showed moderate to good affinities for the 5-

HT₄R with low nanomolar K_i demonstrating that the introduction of a large iodine atom on the aromatic ring is compatible with a conserved interaction with the receptor (Table 1). We could however noticed that the iodination is detrimental to 5-HT₄R affinity for the two ketones **18a-b** (K_i = 86 and 521 nM respectively) which were less potent ligands than their non-iodinated precursors **14a-b** (K_i = 24.7 and 39.9 nM respectively). On the other hand introduction of iodine on the amides **18c-f** generally improves their affinities for the 5-HT₄R and **18c** was found to be the most potent ligand with a K_i of 8.64 nM (Table 1). Concerning the other pharmacomodulations, the different alkoxy chains developed did not influence the 5-HT₄R binding and the methylenecyclohexyl linker was found superior than the propyl one (**18e** vs **18f**). Concerning the influence of these modulations on the lipophilicity, the amide linker appears again to be the most appropriate with calculated or measured LogP between 3.5 and 5.2 for **18f** and **18d** respectively. The amide derivatives were also identified to be able to cross the BBB in a predictive PAMPA assay since all compounds **18c-f** could be considered as CNS + compounds. Based on this positive preliminary studies compound **18c** and **18d**, the most potent compounds, were designated for the development of [¹²⁵I]-ligand in order to evaluate their efficiency as SPECT radiotracers.

Thus, the radioactive labeling of compounds **17a-b** was performed and the resulting [¹²⁵I]**18c** and [¹²⁵I]**18d** were evaluated as radiotracers *in vivo*, *ex vivo* and *in vitro*. After injection of each radioligands in rat, *in vivo* SPECT imaging showed activity distribution in the brain and validated their ability to cross the BBB. Mdr1a KO rats were used for the *in vivo* experiments in order to evaluate only the ability of the [¹²⁵I]**18c** and [¹²⁵I]**18d** tracers to specifically bind to 5-HT₄R regardless of the Mdr1a expression. Autoradiograms obtained for *ex vivo* imaging showed an inability to obtain specific labeling in regions known to contain high densities of 5-HT₄R. In order to address their 5-HT₄R specific binding capacity, *in vitro* competition experiments with [¹²⁵I]**1** were performed. The selective and specific antagonist radioligand was co-administered with **18c** and **18d**. Increasing concentrations of both iodinated derivatives showed a decrease in the 5-HT₄R-specific radioactivity, while increasing the concentration to 10 μM led to the almost complete abolishment of the signal.

4. Conclusion

In conclusion we have demonstrated that the modulation of the ester function of the reference 5-HT₄R ligands and their replacement by an amide or a ketone function do not affect the ability of the ligand to cross the blood-brain barrier. Among the synthesized derivatives, the most promising results were obtained with the amide analog **18c** ($K_i = 8.64$ nM) which was able to bind to the 5-HT₄R *in vitro* as demonstrated by the displacement of [¹²⁵I]**1**. However the non-specific interactions of [¹²⁵I]**18c-d** with other brain regions might be explained by a fast metabolism or by their high lipophilicities which would be optimized in a future study.

5. Experimental Section

5.1 Chemistry. All commercially available compounds were used without further purification. Melting points were determined on a Kfler apparatus. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ on aluminium plates (Merck) and visualized with UV light (254 nm). Flash chromatography was conducted on a VWR SPOT II Essential instrument with silica gel 60 (40-63m). Column's size and flow rate were used according to manufacturer's recommendation. NMR spectra were recorded at 400 or 500 MHz (Bruker Avance III 400/500 MHz) for ¹H NMR, at 100 or 125 MHz for ¹³C NMR and at 376.1 MHz for ¹⁹F in chloroform-*d*, methanol-*d*₄ or DMSO-*d*₆ with chemical shift (δ) given in parts per million (ppm) relative to TMS as ¹H and ¹³C NMR internal standard and CFCI₃ as ¹⁹F NMR reference standard, and recorded at 295K. The following abbreviations are used to describe peak splitting patterns when appropriate: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, dt = doublet of triplet. Coupling constants *J* are reported in hertz units (Hz). Infrared spectra (IR) were obtained on a PERKIN-ELMER FT-IR spectrometer and are reported in terms of frequency of absorption (cm⁻¹) using KBr discs. High-resolution mass spectra (HRMS) were obtained by electronic impact (HRMS/EI), or by electrospray (HRMS/ESI) on a Bruker maXis mass spectrometer. LC-MS (ESI) analyses were realized with Waters Alliance 2695 as separating module using the following gradients: A (95%)/B (5%) to A (5%)/B (95%) in 4.00min. This ratio was hold during 1.50 min before return to initial conditions in 0.50 min. Initial conditions were then maintained for 2.00 min (A = H₂O, B = CH₃CN ; each containing HCOOH : 0.1 %; column XBridge C18 2.5 m/4.6x50 mm; flow rate 0.8 mL/min). MS were obtained on a SQ detector by positive ESI. Mass spectrum data are reported as m/z.

Methyl 4-amino-2-hydroxybenzoate (9). To a stirred solution of 4-amino-2-hydroxybenzoic acid (2.0 g, 13.1 mmol, 1.0 eq.) in MeOH (40 mL) was added dropwise concentrated aqueous solution of H₂SO₄ (2.8 mL) and the resulting mixture was refluxed for 16 h. After cooling to room temperature, it was neutralized with saturated aqueous NaHCO₃ solution until no further gas evolution was observed and the mixture was concentrated *in vacuo*. The residue was dissolved in water and extracted several times with EtOAc. The combined organic extracts were dried over MgSO₄ and concentrated under reduced pressure to afford (9), as a brown solid (1.97 g, 90% yield); mp 113 °C (litt.: 115 °C [30]); ¹H NMR (CDCl₃, 400 MHz) δ 10.95 (br s, 1H, OH), 7.60 (d, ³J = 8.9 Hz, 1H), 6.14 (m, 2H), 4.15 (br s, 2H, NH₂), 3.86 (s, 3H); ¹³C NMR (CDCl₃, 100 MHz) δ 170.6, 163.6, 153.5, 131.7, 106.9, 103.0, 100.7, 51.8; IR (neat, cm⁻¹) ν 3475, 3381, 3249, 3025, 2952, 2851, 1642, 1437, 1356, 1283, 780; MS *m/z* [M+H]⁺ 168.17.

Methyl 4-amino-2-ethoxybenzoate (10a). To a stirred solution of Methyl 4-amino-2-hydroxybenzoate (9) (500 mg, 3.0 mmol, 1.0 eq.) in DMF (25 mL) were added K₂CO₃ (829 mg, 6.0 mmol, 2.0 eq.) and Iodoethane (288 μL, 3.6 mmol, 1.2 eq.), then the resulting mixture was stirred at 70 °C overnight. After cooling to room temperature, the mixture was concentrated *in vacuo*. The crude was dissolved with EtOAc, then the organic layer was washed with brine, dried over MgSO₄ and concentrated *in vacuo*. The crude was purified by chromatography on silica gel column (DCM/EtOAc, gradient 100:0 to 90:10) and concentrated under reduced pressure to afford (10a), as a white solid (360 mg, 61% yield); mp 104 °C; ¹H NMR (CDCl₃, 400 MHz) δ 7.70 (d, ³J = 8.6 Hz, 1H), 6.18 (dd, ³J = 8.6 Hz, ⁴J = 2.2 Hz, 1H), 6.15 (d, ⁴J = 2.2 Hz, 1H), 4.13 (br s, 2H, NH₂), 3.99 (q, ³J = 7.1 Hz, 2H), 3.79 (s, 3H), 1.42 (t, ³J = 7.1 Hz, 3H); ¹³C NMR (CDCl₃, 100 MHz) δ 166.4, 161.2, 152.2, 134.2, 109.0, 106.5, 98.8, 64.4, 51.4, 14.8; IR (neat, cm⁻¹) ν 3500, 3356, 3223, 2978, 2947, 1694, 1608, 1254, 1038, 811; HRMS (ESI) *m/z* calcd. for C₁₀H₁₄NO₃ [M+H]⁺ 196.0968, found 196.0965.

Methyl 4-amino-2-(2-fluoroethoxy)benzoate (10b). To a stirred solution of Methyl 4-amino-2-hydroxybenzoate (9) (500 mg, 3.0 mmol, 1.0 eq.) in DMF (25 mL) were added K₂CO₃ (829 mg, 6.0 mmol, 2.0 eq.) and 2-fluoroethyl 4-methylbenzenesulfonate (720 mg, 3.3 mmol, 1.1 eq.) and the resulting mixture was stirred at 110 °C for 2 h. After cooling to room temperature, the mixture was concentrated *in vacuo*. The crude was dissolved with EtOAc, then the organic layer

was washed with brine, dried over MgSO_4 and concentrated *in vacuo*. The crude was purified by chromatography on silica gel column (DCM/EtOAc, gradient 100:0 to 90:10) and concentrated under reduced pressure to afford (**10b**), as a white solid (397 mg, 62% yield) ; mp 105 °C; ^1H NMR (CDCl_3 , 400 MHz) δ 7.74 (d, $^3J = 8.5$ Hz, 1H), 6.28 (dd, $^3J = 8.5$ Hz, $^4J = 2.2$ Hz, 1H), 6.20 (d, $^4J = 2.2$ Hz, 1H), 4.78 (dt, $^2J = 47.4$ Hz, $^3J = 4.2$ Hz, 2H), 4.23 (dt, $^3J = 27.0$ Hz, $^3J = 4.3$ Hz, 2H), 4.07 (br s, 2H, NH_2), 3.82 (s, 3H); ^{13}C NMR (CDCl_3 , 100 MHz) δ 166.2, 160.8, 152.0, 134.4, 110.1, 107.6, 100.3, 82.1 (d, $^1J = 171.0$ Hz), 68.8 (d, $^2J = 20.7$ Hz), 51.6; ^{19}F NMR (CDCl_3 , 376 MHz) δ -223.6 (tt, $^2J = 47.4$ Hz, $^3J = 27.0$ Hz); IR (neat, cm^{-1}) ν 3497, 3370, 3236, 2965, 2947, 1690, 1607, 1254, 1087, 776; HRMS (ESI) m/z calcd. for $\text{C}_{10}\text{H}_{13}\text{FNO}_3$ $[\text{M}+\text{H}]^+$ 214.0874, found 214.0872.

Representative procedure (d) for the synthesis of 11a-b. To a stirred solution of methyl ester derivatives (1.0 eq.) in EtOH (10 mL/mmol) was added an aqueous 1N NaOH solution (10.0 eq.) under nitrogen atmosphere at room temperature. The resulting mixture was stirred overnight at room temperature, and then concentrated *in vacuo* to remove EtOH. The residue was diluted with water. The aqueous layer was acidified by addition of hydrochloric acid until acidic pH, and extracted several times with ethyl acetate. The combined organic extract was washed with brine, dried over MgSO_4 and concentrated under reduced pressure to give the title compounds **11a-b**.

4-amino-2-ethoxybenzoic acid (11a). The compound was prepared from Methyl 4-amino-2-ethoxybenzoate (**10a**) (870 mg, 4.5 mmol) according to *procedure (d)* and was obtained as a pale yellow solid (756 mg, 94% yield); mp 146 °C (litt.: 152-154 °C [31]); ^1H NMR ($\text{MeOD}-d_4$, 400 MHz) δ 7.70 (d, $^3J = 8.6$ Hz, 1H), 6.33 (d, $^4J = 2.0$ Hz, 1H), 6.29 (dd, $^3J = 8.6$ Hz, $^4J = 2.0$ Hz, 1H), 4.21 (q, $^3J = 7.0$ Hz, 2H), 1.47 (t, $^3J = 7.0$ Hz, 3H); ^{13}C NMR ($\text{MeOD}-d_4$, 100 MHz) δ 169.4, 161.8, 156.9, 135.5, 108.3, 106.1, 98.2, 66.1, 14.8; IR (neat, cm^{-1}) ν 3435, 3353, 3242, 2982, 2930, 2851, 1690, 1604, 1404, 1273, 1197, 1029; MS m/z $[\text{M}+\text{H}]^+$ 182.42.

4-amino-2-(2-fluoroethoxy)benzoic acid (11b). The compound was prepared from Methyl 4-amino-2-(2-fluoroethoxy)benzoate (**10b**) (940 mg, 4.4 mmol) according to *procedure (d)* and was obtained as a pale yellow solid (840 mg, 96% yield); mp 124 °C; ^1H NMR ($\text{MeOD}-d_4$, 400 MHz) δ 7.70 (d, $^3J = 8.4$ Hz, 1H), 6.34-6.30 (m, 2H), 4.79 (dt, $^2J = 47.7$ Hz, $^3J = 4.1$ Hz, 2H),

4.34 (dt, $^3J = 27.9$ Hz, $^3J = 4.1$ Hz, 2H); ^{13}C NMR (MeOD- d_4 , 100 MHz) δ 169.3, 161.7, 156.8, 135.6, 108.6, 106.8, 98.8, 82.7 (d, $^1J = 167.7$ Hz), 69.8 (d, $^2J = 19.8$ Hz); ^{19}F NMR (MeOD- d_4 , 376 MHz) δ -226.22 (tt, $^2J = 47.7$ Hz, $^3J = 27.9$ Hz); IR (neat, cm^{-1}) ν 3490, 3365, 3282, 3240, 2984, 2949, 1697, 1609, 1388, 1274, 1196, 1039, 884; HRMS (ESI) m/z calcd. for $\text{C}_9\text{H}_{11}\text{FNO}_3$ $[\text{M}+\text{H}]^+$ 200.0717, found 200.0714.

Representative procedure (e) for the synthesis of 12a-b. To a solution of benzoic acid derivatives (1.0 eq.) in dry THF (10 mL/mmol) was added CDI (1.1 eq.) and the resulting mixture was stirred at room temperature for 15 h. Then Potassium 3-ethoxy-3-oxopropanoate (1.2 eq.) and MgCl_2 (1.2 eq.) were added portion-wise. The reaction mixture was stirred at 40 °C for 24 h. After removal of the solvent, the residue was dissolved with EtOAc, and washed with a saturated aqueous NaHCO_3 solution then brine. The organic layer was dried (MgSO_4) and concentrated in vacuo. Chromatographic separation gave the title compounds.

Ethyl 3-(4-amino-2-ethoxyphenyl)-3-oxopropanoate (12a). The compound was prepared from 4-amino-2-ethoxybenzoic acid (**11a**) (750 mg, 4.1 mmol) according to *procedure (e)*. After a purification by chromatography on silica gel column (cyclohexane/EtOAc, gradient 100:0 to 70:30), the compound (**12a**) was obtained as a white solid (233 mg, 23% isolated yield); mp 119 °C; ^1H NMR (CDCl_3 , 400 MHz) δ 7.82 (d, $^3J = 8.6$ Hz, 1H), 6.25 (dd, $^3J = 8.6$ Hz, $^4J = 2.1$ Hz, 1H), 6.10 (d, $^4J = 2.0$ Hz, 1H), 4.18 (q, $^3J = 7.1$ Hz, 2H), 4.05 (q, $^3J = 7.0$ Hz, 2H), 3.94 (s, 2H), 1.45 (t, $^3J = 7.0$ Hz, 3H), 1.24 (t, $^3J = 7.1$ Hz, 3H); ^{13}C NMR (CDCl_3 , 100 MHz) δ 190.9, 169.2, 161.3, 153.2, 133.7, 117.1, 107.4, 97.1, 64.1, 60.9, 50.7, 14.7, 14.3; IR (neat, cm^{-1}) ν 3477, 3381, 3244, 2977, 2938, 2889, 1720, 1594, 1457, 1338, 1204, 1017, 832; HRMS (ESI) m/z calcd. for $\text{C}_{13}\text{H}_{18}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 252.1230, found 252.1227.

Ethyl 3-[4-amino-2-(2-fluoroethoxy)phenyl]-3-oxopropanoate (12b). The compound was prepared from 4-amino-2-(2-fluoroethoxy)benzoic acid (**11b**) (840 mg, 4.21 mmol) according to *procedure (e)*. After a purification by chromatography on silica gel column (cyclohexane/EtOAc, gradient 100:0 to 50:50), the compound (**12b**) was obtained as a white solid (406 mg, 36% isolated yield); mp 117 °C; ^1H NMR (CDCl_3 , 400 MHz) δ 7.85 (d, $^3J = 8.6$ Hz, 1H), 6.30 (dd, $^3J = 8.6$ Hz, $^4J = 2.1$ Hz, 1H), 6.09 (d, $^4J = 2.0$ Hz, 1H), 4.79 (dt, $^2J = 47.3$ Hz, $^3J = 4.1$ Hz, 2H),

4.24 (dt, $^3J = 27.6$ Hz, $^3J = 4.2$ Hz, 2H), 4.18 (q, $^3J = 7.1$ Hz, 2H), 3.96 (s, 2H), 1.24 (t, $^3J = 7.1$ Hz, 3H); ^{13}C NMR (CDCl_3 , 100 MHz) δ 190.8, 169.1, 160.5, 153.2, 133.8, 117.2, 108.1, 97.3, 81.5 (d, $^1J = 170.8$ Hz), 67.6 (d, $^2J = 20.3$ Hz), 61.0, 50.6, 14.2; ^{19}F NMR (CDCl_3 , 376 MHz) δ -223.19 (tt, $^2J = 47.4$ Hz, $^3J = 27.4$ Hz); IR (neat, cm^{-1}) ν 3471, 3375, 3244, 2990, 2962, 2927, 2854, 1710, 1612, 1446, 1341, 1208, 1015, 822; HRMS (ESI) m/z calcd. for $\text{C}_{13}\text{H}_{17}\text{FNO}_4$ $[\text{M}+\text{H}]^+$ 270.1136, found 270.1132.

Representatives procedures (f) and (g) for the synthesis of 13a-b. To a solution of β -keto ester derivatives (1.0 eq.) in DMF (10 mL/mmol) were added K_2CO_3 (2.0 eq.) and *Tert*-butyl 4-(iodomethyl)piperidine-1-carboxylate (1.2 eq.). The resulting mixture was stirred at room temperature for 48 h then concentrated *in vacuo*. The residue was dissolved with EtOAc and washed with brine. The organic layer was dried (MgSO_4) and concentrated in vacuo. To a stirred solution of residue (1.0 eq.), used without any purification, in a mixture of EtOH/ H_2O 5:1 (24 mL/mmol) was added KOH (4.5 eq.) and the resulting mixture was refluxed for 5 h. After removal of the solvent, EtOAc was added. The organic layer was washed with brine, dried over MgSO_4 and concentrated in vacuo. The residue was purified by chromatography on silica gel to give the title compounds **13a-b**.

***Tert*-butyl 4-[3-(4-amino-2-ethoxyphenyl)-3-oxopropyl]piperidine-1-carboxylate (13a).** The compound was prepared from Ethyl 3-(4-amino-2-ethoxyphenyl)-3-oxopropanoate (**12a**) (249 mg, 1.0 mmol) according to *procedures (f) and (g)*. After a purification by chromatography on silica gel column (cyclohexane/EtOAc, gradient 100:0 to 60:40), the compound (**13a**) was obtained as a yellow oil (279 mg, 74% isolated yield over 2 steps); ^1H NMR (CDCl_3 , 400 MHz) δ 7.69 (d, $^3J = 8.5$ Hz, 1H), 6.23 (dd, $^3J = 8.5$ Hz, $^4J = 2.1$ Hz, 1H), 6.12 (d, $^4J = 2.0$ Hz, 1H), 4.05 (m, 4H), 2.96 (t, $^3J = 7.3$ Hz, 2H), 2.66 (m, 2H), 1.67-1.58 (m, 4H), 1.45 (t, $^3J = 7.0$ Hz, 3H), 1.44 (s, 9H), 1.41 (m, 1H), 1.10 (m, 2H); ^{13}C NMR (CDCl_3 , 100 MHz) δ 200.1, 160.7, 155.0, 152.2, 133.2, 118.6, 107.2, 97.6, 79.3, 63.9, 44.1 (2C), 40.9, 36.0, 32.2 (2C), 31.3, 28.6 (3C), 15.0; IR (neat, cm^{-1}) ν 3445, 3356, 3239, 2979, 2931, 2858, 1675, 1646, 1594, 1277, 1037, 818; HRMS (ESI) m/z calcd. for $\text{C}_{21}\text{H}_{33}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 377.2435, found 377.2435.

***Tert*-butyl 4-{3-[4-amino-2-(2-fluoroethoxy)phenyl]-3-oxopropyl}piperidine-1-carboxylate (13b).** The compound was prepared from Ethyl 3-[4-amino-2-(2-fluoroethoxy)phenyl]-3-oxopropanoate (**12b**) (400 mg, 1.49 mmol) according to *procedures (f)* and *(g)*. After a purification by chromatography on silica gel column (cyclohexane/EtOAc, gradient 100:0 to 60:40), the compound (**13b**) was obtained as a yellow oil (414 mg, 70% isolated yield over 2 steps); mp 119 °C; ¹H NMR (CDCl₃, 400 MHz) δ 7.66 (d, ³J = 8.5 Hz, 1H), 6.23 (dd, ³J = 8.5 Hz, ⁴J = 1.9 Hz, 1H), 6.08 (d, ⁴J = 1.9 Hz, 1H), 4.72 (dt, ²J = 47.5 Hz, ³J = 5.3 Hz, 2H), 4.32 (br s, 2H, NH₂), 4.16 (dt, ³J = 5.3 Hz, ³J = 28.0 Hz, 2H), 4.02 (m, 2H), 2.95 (t, ³J = 7.4 Hz, 2H), 2.63 (m, 2H), 1.65-1.55 (m, 4H), 1.41 (s, 9H), 1.37 (m, 1H), 1.05 (m, 2H); ¹³C NMR (CDCl₃, 100 MHz) δ 199.9, 160.0, 154.9, 152.7, 133.1, 118.0, 107.6, 97.4, 81.6 (d, ¹J = 170.9 Hz), 79.4, 67.4 (d, ²J = 19.7 Hz), 43.7 (2C), 40.8, 35.8, 32.0 (2C), 31.1, 28.4 (3C); ¹⁹F NMR (CDCl₃, 376 MHz) δ -223.28 (tt, ²J = 47.5 Hz, ³J = 27.8 Hz); IR (neat, cm⁻¹) ν 3450, 3355, 3239, 2926, 2858, 1677, 1641, 1595, 1277, 1062; HRMS (ESI) *m/z* calcd. for C₂₁H₃₂FN₂O₄ [M+H]⁺ 395.2341, found 395.2341.

Representatives procedures (h) and (i) for the synthesis of 14a-b. To a stirred solution of *Tert*-butyl piperidine-1-carboxylate derivatives (1.0 eq.) in DCM (20 mL/mmol) was added TFA (2 mL/mmol). The resulting mixture was stirred at room temperature for 1 h. Removal of the solvent under vacuum afforded the crude product, which was directly engaged in the next step. The residue obtained (1.0 eq.) was dissolved in DMF (10 mL/mmol) and Bromomethylcyclohexane (1.1 eq.) and K₂CO₃ (10.0 eq.) were added. The resulting mixture was stirred at 110 °C for 5 h, and then concentrated *in vacuo*. Ethyl acetate was added, the organic layer was washed several times with brine, dried over MgSO₄ and concentrated *in vacuo*. The crude was purified by chromatography on silica gel column and concentrated under reduced pressure to afford the corresponding alkylated compounds **14a-b**.

1-(4-amino-2-ethoxyphenyl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one (14a). The compound was prepared from *Tert*-butyl 4-[3-(4-amino-2-ethoxyphenyl)-3-oxopropyl]piperidine-1-carboxylate (**13a**) (280 mg, 0.74 mmol) according to *procedures (h)* and *(i)*. After a purification by chromatography on silica gel column (cyclohexane/EtOAc, gradient 100:0 to 20:80), the compound (**14a**) was obtained as a yellow solid (159 mg, 58% isolated yield

over 2 steps); mp 84 °C; ¹H NMR (CDCl₃, 400 MHz) δ 7.68 (d, ³J = 8.5 Hz, 1H), 6.23 (dd, ³J = 8.5 Hz, ⁴J = 2.1 Hz, 1H), 6.12 (d, ⁴J = 2.0 Hz, 1H), 4.09 (br s, 2H, NH₂), 4.05 (q, ³J = 7.0 Hz, 2H), 3.02 (m, 2H), 2.95 (t, ³J = 7.4 Hz, 2H), 2.28 (m, 2H), 2.07 (m, 2H), 1.79-1.59 (m, 9H), 1.56 (m, 1H), 1.45 (t, ³J = 7.0 Hz, 3H), 1.38 (m, 1H), 1.27-1.08 (m, 5H), 0.90 (m, 2H); ¹³C NMR (CDCl₃, 100 MHz) δ 200.1, 160.8, 152.2, 133.2, 118.5, 107.1, 97.6, 65.4, 63.9, 54.2 (2C), 41.0, 35.2, 34.7, 32.1 (2C), 31.2 (2C), 31.0, 26.6, 26.1 (2C), 15.0; IR (neat, cm⁻¹) ν 3445, 3349, 3242, 2919, 2846, 1637, 1587, 1454, 1273, 1036, 980, 827; HRMS (ESI) *m/z* calcd. for C₂₃H₃₇N₂O₂ [M+H]⁺ 373.2850, found 373.2850.

1-[4-amino-2-(2-fluoroethoxy)phenyl]-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one (14b). The compound was prepared from *Tert*-butyl 4-[3-[4-amino-2-(2-fluoroethoxy)phenyl]-3-oxopropyl]piperidine-1-carboxylate (**13b**) (240 mg, 0.61 mmol) according to *procedures (h)* and *(i)*. After a purification by chromatography on silica gel column (cyclohexane/EtOAc, gradient 100:0 to 20:80), the compound (**14b**) was obtained as a yellow solid (102 mg, 43% isolated yield over 2 steps); mp 72 °C; ¹H NMR (CDCl₃, 400 MHz) δ 7.70 (d, ³J = 8.5 Hz, 1H), 6.27 (dd, ³J = 8.5 Hz, ⁴J = 2.0 Hz, 1H), 6.10 (d, ⁴J = 2.0 Hz, 1H), 4.77 (dt, ²J = 47.4 Hz, ³J = 4.1 Hz, 2H), 4.22 (dt, ³J = 4.1 Hz, ³J = 27.6 Hz, 2H), 4.10 (br s, 2H, NH₂), 2.96 (t, ³J = 7.6 Hz, 2H), 2.83 (m, 2H), 2.06 (d, ³J = 7.0 Hz, 2H), 1.82-1.57 (m, 11H), 1.46 (m, 1H), 1.27-1.08 (m, 6H), 0.84 (m, 2H); ¹³C NMR (CDCl₃, 100 MHz) δ 200.3, 160.0, 152.1, 133.3, 118.9, 107.8, 97.8, 81.7 (d, ¹J = 171.2 Hz), 67.5 (d, ²J = 20.2 Hz), 66.4, 54.7 (2C), 41.3, 36.0, 35.4, 32.5 (2C), 32.3 (2C), 31.4, 26.9, 26.4 (2C); ¹⁹F NMR (CDCl₃, 376 MHz) δ -223.3 (tt, ²J = 47.1 Hz, ³J = 27.8 Hz); IR (neat, cm⁻¹) ν 3429, 3347, 3240, 2921, 2849-2768, 1637, 1591, 1443, 1274; HRMS (ESI) *m/z* calcd. for C₂₃H₃₆FN₂O₂ [M+H]⁺ 391.2755, found 391.2755.

Representative procedure (j) for the synthesis of 15a-b. To a stirred solution of piperidine-4-carboxamide (1.0 eq.) in EtOH (2mL/mmol) were added K₂CO₃ (2.0 eq.) and alkyl-halogenated compound (1.1-1.25 eq.) and the resulting mixture was refluxed for 15-24 h. After cooling to room temperature, the mixture was concentrated *in vacuo*. The crude was dissolved with CHCl₃, then the organic layer was washed with brine, dried over MgSO₄ and concentrated *in vacuo*. The residue obtained was precipitated with petroleum ether and filtered to afford alkylated compound (92-93% yields).

1-propylpiperidine-4-carboxamide (15a). The compound was prepared from Piperidine-4-carboxamide (6.0 g, 46.8 mmol) and 1-iodopropane (9.94 g, 58.5 mmol) according to *procedure (j)*, with a reflux for 15 h, and was obtained as a white solid (7.4 g, 92% yield); mp 162 °C; ^1H NMR (CDCl_3 , 400 MHz) δ 5.49 (br s, 2H, NH_2), 2.97 (m, 2H), 2.29-2.25 (m, 2H), 2.19-2.11 (m, 1H), 1.96-1.88 (m, 4H), 1.80-1.68 (m, 2H), 1.54-1.45 (m, 2H), 0.89 (t, $^3J = 7.4$ Hz, 3H); ^{13}C NMR (CDCl_3 , 100 MHz) δ 177.4, 61.0, 53.4 (2C), 43.1, 29.2 (2C), 20.3, 12.1; IR (neat, cm^{-1}) ν 3382, 3194, 2953, 2931, 2872, 2809-2680, 1653, 1425, 1143, 675; HRMS (ESI) m/z calcd. for $\text{C}_9\text{H}_{19}\text{N}_2\text{O}$ $[\text{M}+\text{H}]^+$ 171.1492, found 171.1489.

1-(cyclohexylmethyl)piperidine-4-carboxamide (15b). The compound was prepared from Piperidine-4-carboxamide (2.92 g, 22.8 mmol) and Bromomethylcyclohexane (4.44 g, 25.1 mmol) according to *procedure (j)*, with a reflux for 24 h, and was obtained as white crystals (4.77 g, 93% yield); mp 178 °C; ^1H NMR (CDCl_3 , 500 MHz) δ 2.89 (m, 2H), 2.09 (m, 1H), 2.08 (d, $^3J = 7.1$ Hz, 2H), 1.91-1.83 (m, 4H), 1.76-1.64 (m, 7H), 1.46 (m, 1H), 1.24-1.10 (m, 3H), 0.84 (m, 2H); ^{13}C NMR (CDCl_3 , 126 MHz) δ 118.0, 66.0, 53.8 (2C), 43.2, 35.3, 32.1 (2C), 29.1 (2C), 26.9, 26.3 (2C); IR (neat, cm^{-1}) ν 3387, 3184, 2939, 2922, 2850, 1648, 1455, 1448, 1126, 635; HRMS (ESI) m/z calcd. for $\text{C}_{13}\text{H}_{25}\text{N}_2\text{O}$ $[\text{M}+\text{H}]^+$ 225.1961, found 225.1959.

Representative procedure (k) for the synthesis of 16a-b. To a stirred solution of alkylated piperidine-4-carboxamide (1.0 eq.) in anhydrous THF (5mL/mmol) at 0 °C under nitrogen atmosphere was carefully added LiAlH_4 (3.0 eq.). The resulting mixture was stirred at room temperature for 1-2 h and then refluxed for 3 h. The reaction was then slowly quenched at 0 °C with n mL of water (n mL for n grams of LiAlH_4 used), n mL of an aqueous 15% NaOH solution followed by $3n$ mL of water to afford a granular inorganic precipitate. The solution was filtered over a pad of celite, the solid was rinsed with diethyl ether and EtOAc, then the filtrate was concentrated under reduced pressure to give reduced compound (71-96% yields).

(1-propyl-4-piperidyl)methanamine (16a). The compound was prepared from 1-propyl piperidine-4-carboxamide (**15a**) (0.2 g, 1.17 mmol) according to *procedure (k)*, with a stirring at room temperature for 1 h and a reflux for 3 h, and was obtained as a yellow oil (0.13 g, 71%

yield); ^1H NMR (CDCl_3 , 400 MHz) δ 2.93 (m, 2H), 2.55 (d, $^3J = 5.9$ Hz, 2H), 2.25 (m, 2H), 1.90-1.83 (m, 2H), 1.70-1.44 (m, 5H), 1.23-1.17 (m, 2H), 0.86 (t, $^3J = 7.3$ Hz, 3H); ^{13}C NMR (CDCl_3 , 126 MHz) δ 61.2, 53.8 (2C), 48.2, 39.5, 30.0 (2C), 20.3, 12.9; IR (neat, cm^{-1}) ν 3378, 2956, 2930, 2874-2735, 1539, 1482, 1309, 1141.

[1-(cyclohexylmethyl)-4-piperidyl]methanamine (16b). The compound was prepared from 1-(cyclohexylmethyl)piperidine-4-carboxamide (**15b**) (3.0 g, 13.4 mmol) according to *procedure (k)*, with a stirring at room temperature for 2 h and a reflux for 3 h, and was obtained as a white solid (2.70 g, 96% yield); mp 83 °C; ^1H NMR (CDCl_3 , 500 MHz) δ 2.91 (br s, 2H, NH_2), 2.85 (m, 2H), 2.56 (d, $^3J = 6.4$ Hz, 2H), 2.07 (d, $^3J = 7.0$ Hz, 2H), 1.81 (m, 2H), 1.72 (m, 2H), 1.69-1.61 (m, 5H), 1.45 (m, 1H), 1.30-1.26 (m, 1H), 1.25-1.08 (m, 5H), 0.83 (m, 2H); ^{13}C NMR (CDCl_3 , 126 MHz) δ 66.3, 54.3 (2C), 47.7, 38.9, 35.3, 32.2 (2C), 29.9 (2C), 26.9, 26.3 (2C); IR (neat, cm^{-1}) ν 3400, 3272, 2920, 2850, 1535, 1481, 1300, 1130; HRMS (ESI) m/z calcd. for $\text{C}_{13}\text{H}_{27}\text{N}_2$ $[\text{M}+\text{H}]^+$ 211.2169, found 211.2169.

Representative procedure (l) for the synthesis of 17a-d. To a stirred solution of acid derivative (1.0 eq.) in DMF (5 mL/mmol) were added Et_3N (1.0 eq.), EDC (1.0 eq.), HOBT (1.0 eq.) and amine derivatives (1.0 eq.) under nitrogen atmosphere and the resulting mixture was stirred at room temperature for 18-72 h. After evaporation *in vacuo* to remove DMF, the residue was purified by chromatography on silica gel column and concentrated under reduced pressure to afford expected amide derivative (25-44% isolated yields).

4-amino-*N*-{[1-(cyclohexylmethyl)-4-piperidyl]methyl}-2-ethoxybenzamide (17a). The compound was prepared from [1-(cyclohexylmethyl)-4-piperidyl]methanamine (**16b**) (221 mg, 1.05 mmol) and 4-amino-2-ethoxybenzoic acid (**11a**) (190 mg, 1.05 mmol) according to *procedure (l)* with a stirring at room temperature for 72 h. After a purification by flash chromatography on silica gel column (DCM/EtOAc, gradient 100:0 to 90:10), the compound (**17a**) was obtained as a yellow oil (110 mg, 28% isolated yield); ^1H NMR (CDCl_3 , 500 MHz) δ 8.01 (d, $^3J = 8.5$ Hz, 1H), 7.95 (br s, 1H, NH), 6.31 (dd, $^3J = 8.5$ Hz, $^4J = 1.5$ Hz, 1H), 6.16 (d, $^4J = 1.6$ Hz, 1H), 4.09 (q, $^3J = 7.0$ Hz, 2H), 3.99 (br s, 2H, NH_2), 3.31 (t, $^3J = 6.2$ Hz, 2H), 2.85 (m, 2H), 2.07 (d, $^3J = 7.0$ Hz, 2H), 1.83 (m, 2H), 1.75-1.63 (m, 7H), 1.55 (m, 1H), 1.48 (t, $^3J = 6.9$

Hz, 3H), 1.45 (m, 1H), 1.34-1.29 (m, 2H), 1.24-1.10 (m, 3H), 0.84 (m, 2H); ^{13}C NMR (CDCl_3 , 126 MHz) δ 165.7, 158.6, 150.9, 134.0, 111.9, 107.7, 98.1, 66.2, 64.4, 54.2 (2C), 45.3, 36.4, 35.4, 32.2 (2C), 30.3 (2C), 26.9, 26.3 (2C), 15.1; IR (neat, cm^{-1}) ν 3402, 3340, 3229, 2921, 2849, 1633, 1601, 1541, 1270, 1198, 1111, 1036; HRMS (ESI) m/z calcd. for $\text{C}_{22}\text{H}_{36}\text{N}_3\text{O}_2$ $[\text{M}+\text{H}]^+$ 374.2802, found 374.2802.

4-amino-*N*-{[1-(cyclohexylmethyl)piperidin-4-yl]methyl}-2-(2-fluoroethoxy)benzamide

(17b). The compound was prepared from [1-(cyclohexylmethyl)-4-piperidyl]methanamine (**16b**) (242 mg, 1.15 mmol) and 4-amino-2-(2-fluoroethoxy)benzoic acid (**11b**) (230 mg, 1.15 mmol) according to *procedure (l)* with a stirring at room temperature for 48 h. After a purification by flash chromatography on silica gel column (DCM/EtOAc, gradient 100:0 to 90:10), the compound (**17b**) was obtained as a yellow oil (200 mg, 44% isolated yield); ^1H NMR (CDCl_3 , 500 MHz) δ 8.03 (d, $^3J = 8.5$ Hz, 1H), 7.79 (br s, 1H, NH), 6.37 (dd, $^3J = 8.5$ Hz, $^4J = 2.1$ Hz, 1H), 6.15 (d, $^4J = 2.1$ Hz, 1H), 4.80 (dt, $^2J = 47.4$ Hz, $^3J = 4.0$ Hz, 2H), 4.28 (dt, $^3J = 27.4$ Hz, $^3J = 4.1$ Hz, 2H), 3.96 (br s, 2H, NH_2), 3.32 (t, $^3J = 6.5$ Hz, 2H), 2.86 (m, 2H), 2.08 (d, $^3J = 7.1$ Hz, 2H), 1.84 (m, 2H), 1.75-1.63 (m, 7H), 1.56 (m, 1H), 1.47 (m, 1H), 1.33 (m, 2H), 1.25-1.12 (m, 3H), 0.84 (m, 2H); ^{13}C NMR (CDCl_3 , 126 MHz) δ 165.4, 157.8, 150.7, 134.3, 112.7, 108.5, 98.3, 81.5 (d, $^1J = 172.1$ Hz), 67.8 (d, $^2J = 19.3$ Hz), 66.3, 54.3 (2C), 45.5, 36.3, 35.4, 32.2 (2C), 30.3 (2C), 27.0, 26.4 (2C); ^{19}F NMR (CDCl_3 , 376 MHz) δ -224.4 (tt, $^2J = 47.3$ Hz, $^3J = 27.3$ Hz); IR (neat, cm^{-1}) ν 3411, 3340, 3222, 2921, 2850, 1635, 1602, 1541, 1283, 1120, 1065, 889; HRMS (ESI) m/z calcd. for $\text{C}_{22}\text{H}_{35}\text{FN}_3\text{O}_2$ $[\text{M}+\text{H}]^+$ 392.2708, found 392.2708.

4-amino-*N*-{[1-(cyclohexylmethyl)piperidin-4-yl]methyl}-2-methoxybenzamide (17c). The compound was prepared from [1-(cyclohexylmethyl)-4-piperidyl]methanamine (**16b**) (975 mg, 4.63 mmol) and commercial 4-amino-2-methoxybenzoic acid (**11c**) (774 mg, 4.63 mmol) according to *procedure (l)* with a stirring at room temperature for 48 h. After a purification by flash chromatography on silica gel column (gradient: DCM to DCM/EtOAc 90:10), the compound (**17c**) was obtained as a white powder (280 mg, 17% isolated yield); ^1H NMR (CDCl_3 , 500 MHz) δ 8.02 (d, $^3J = 8.5$ Hz, 1H), 7.79 (br s, 1H, NH), 6.34 (dd, $^3J = 8.5$ Hz, $^4J = 2.1$ Hz, 1H), 6.20 (d, $^4J = 2.1$ Hz, 1H), 3.98 (br s, 2H, NH_2), 3.90 (s, 3H), 3.31 (t, $^3J = 6.3$ Hz, 2H), 2.87 (m, 2H), 2.10 (d, $^3J = 7.0$ Hz, 2H), 1.86 (m, 2H), 1.76-1.64 (m, 7H), 1.58 (m, 1H), 1.47

(m, 1H), 1.34 (m, 2H), 1.25-1.10 (m, 3H), 0.85 (m, 2H); ^{13}C NMR (CDCl_3 , 126 MHz) δ 165.6, 159.2, 150.9, 134.1, 112.1, 107.8, 97.4, 66.2, 55.9, 54.2 (2C), 45.3, 36.4, 35.4, 32.2 (2C), 30.2 (2C), 26.9, 26.3 (2C); IR (neat, cm^{-1}) ν 3408, 3342, 3224, 2921, 2849, 1638, 1601, 1538, 1505, 1281, 1208; HRMS (ESI) m/z calcd. for $\text{C}_{21}\text{H}_{34}\text{N}_3\text{O}_2$ $[\text{M}+\text{H}]^+$ 360.2646, found 360.2644.

4-amino-2-methoxy-*N*-[(1-propyl-4-piperidyl)methyl]benzamide (17d). The compound was prepared from (1-propyl-4-piperidyl)methanamine (**16a**) (562 mg, 3.60 mmol) and commercial 4-amino-2-methoxybenzoic acid (**11c**) (600 mg, 3.60 mmol) according to *procedure (l)* with a stirring at room temperature for 18 h. After a first purification by chromatography on silica gel column (DCM/EtOAc, gradient 100:0 to 90:10), the crude was finally purified by C18 reversed phase column ($\text{H}_2\text{O}/\text{ACN}$, gradient 100:0 to 90:10) to separate expected product and side product of peptide coupling (similar polarity on silica gel), and concentrated under reduced pressure to afford the compound (**17d**) as a white solid (275 mg, 25% isolated yield); mp 134 °C; ^1H NMR ($\text{MeOD}-d_4$, 500 MHz) δ 7.71 (d, $^3J = 8.5$ Hz, 1H), 6.35 (d, $^4J = 2.0$ Hz, 1H), 6.30 (dd, $^3J = 8.5$ Hz, $^4J = 2.0$ Hz, 1H), 3.91 (s, 3H), 3.55 (m, 2H), 3.36 (m, 2H), 3.00 (m, 2H), 2.96 (m, 2H), 1.99 (m, 2H), 1.93 (m, 1H), 1.75 (m, 2H), 1.55 (m, 2H), 1.01 (t, $^3J = 7.4$ Hz, 3H); ^{13}C NMR ($\text{MeOD}-d_4$, 126 MHz) δ 168.9, 161.1, 155.2, 133.9, 110.1, 107.9, 97.6, 59.6, 56.2, 53.5 (2C), 44.7, 35.5, 28.3 (2C), 18.7, 11.2; IR (neat, cm^{-1}) ν 3394, 3210, 2937, 2655, 2534, 1624, 1600, 1543, 1505, 1282, 1210; HRMS (ESI) m/z calcd. for $\text{C}_{17}\text{H}_{28}\text{N}_3\text{O}_2$ $[\text{M}+\text{H}]^+$ 306.2176, found 306.2173.

Representative procedure (m) for iodination with standard conditions of radiolabeling. To a stirred solution of amide derivative (1.0 eq.) in peracetic acid (acetic acid/30% H_2O_2 2:1, 50 mL/mmol) was added NaI (1.1 eq.) (typical brown iodine color was observed) and the resulting mixture was stirred at room temperature for 1-2 h. The reaction was cooled at 0 °C in an ice-water bath, and were added respectively water and a saturated aqueous $\text{Na}_2\text{S}_2\text{O}_3$ solution. The mixture was then neutralized by addition of an aqueous 2N NaOH solution until basic pH (~10), and extracted several times with DCM. The combined organic extract was dried over MgSO_4 and was concentrated *in vacuo*. The residue was purified by chromatography on silica gel column and concentrated under reduced pressure to afford iodinated compound (18-71% isolated yields).

1-(4-amino-2-ethoxy-5-iodophenyl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one

(18a). The compound was prepared from 1-(4-amino-2-ethoxyphenyl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one (**14a**) (44 mg, 0.12 mmol) according to *procedure (m)* with a stirring at room temperature for 1 h. After a purification by flash chromatography on silica gel column (DCM/MeOH, gradient 100:0 to 95:5), the compound (**18a**) was obtained as a yellow solid (11 mg, 18% isolated yield); mp 98 °C; ¹H NMR (CDCl₃, 400 MHz) δ 8.11 (s, 1H), 6.23 (s, 1H), 4.53 (br s, 2H, NH₂), 4.05 (q, ³J = 6.9 Hz, 2H), 3.29 (m, 2H), 2.95 (t, ³J = 7.2 Hz, 2H), 2.57 (m, 2H), 2.40 (m, 2H), 1.88-1.64 (m, 10H), 1.51 (m, 1H), 1.46 (t, ³J = 7.0 Hz, 3H), 1.30-1.09 (m, 5H), 1.01 (m, 2H); ¹³C NMR (CDCl₃, 100 MHz) δ 198.3, 160.7, 151.7, 142.0, 120.3, 97.2, 73.3, 64.7, 64.3, 54.0 (2C), 40.4, 33.9 (2C), 32.0 (2C), 29.8 (3C), 26.1, 25.9 (2C), 14.9; IR (neat, cm⁻¹) ν 3429, 3187, 2924, 2851, 1625, 1575, 1436, 1260, 1211, 1189, 1039; HRMS (ESI) *m/z* calcd. for C₂₃H₃₆IN₂O₂ [M+H]⁺ 499.1816, found 499.1817.

1-[4-amino-2-(2-fluoroethoxy)-5-iodophenyl]-3-[1-(cyclohexylmethyl)-4-piperidyl] propan-1-one (18b).

The compound was prepared from 1-[4-amino-2-(2-fluoroethoxy)phenyl]-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one (**14b**) (77 mg, 0.20 mmol) according to *procedure (m)* with a stirring at room temperature for 1 h. After a purification by flash chromatography on silica gel column (cyclohexane/EtOAc, gradient 100:0 to 20:80), the compound (**18b**) was obtained as a pale yellow solid (42 mg, 41% isolated yield); mp 129 °C; ¹H NMR (CDCl₃, 400 MHz) δ 8.13 (s, 1H), 6.21 (s, 1H), 4.78 (dt, ²J = 47.4 Hz, ³J = 4.0 Hz, 2H), 4.49 (br s, 2H, NH₂), 4.22 (dt, ³J = 4.1 Hz, ³J = 27.5 Hz, 2H), 2.95 (t, ³J = 7.6 Hz, 2H), 2.83 (m, 2H), 2.07 (d, ³J = 7.0 Hz, 2H), 1.83-1.56 (m, 11H), 1.47 (m, 1H), 1.25-1.09 (m, 6H), 0.85 (m, 2H); ¹³C NMR (CDCl₃, 100 MHz) δ 199.0, 159.8, 151.3, 142.2, 120.9, 97.4, 81.5 (d, ¹J = 171.6 Hz), 74.1, 67.8 (d, ²J = 20.2 Hz), 66.4, 54.7 (2C), 41.2, 36.0, 35.4, 32.4 (2C), 32.3 (2C), 31.3, 27.0, 26.4 (2C); ¹⁹F NMR (CDCl₃, 376 MHz) δ -223.3 (tt, ²J = 47.4 Hz, ³J = 27.4 Hz); IR (neat, cm⁻¹) ν 3459, 3320, 3203, 2920, 2845, 1637, 1569, 1430, 1214, 1071, 899; HRMS (ESI) *m/z* calcd. for C₂₃H₃₅FIN₂O₂ [M+H]⁺ 517.1722, found 517.1722.

4-amino-N-[[1-(cyclohexylmethyl)-4-piperidyl]methyl]-2-ethoxy-5-iodobenzamide (18c).

The compound was prepared from 4-amino-N-[[1-(cyclohexylmethyl)-4-piperidyl]methyl]-2-ethoxybenzamide (**17a**) (50 mg, 0.13 mmol) according to *procedure (m)* with a stirring at room

temperature for 1 h. After a purification by flash chromatography on silica gel column (DCM/MeOH, gradient 100:0 to 90:10), the compound (**18c**) was obtained as a pale yellow solid (46 mg, 71% isolated yield); mp 153 °C; ¹H NMR (CDCl₃, 500 MHz) δ 8.44 (s, 1H), 7.86 (br s, 1H, NH), 6.25 (s, 1H), 4.41 (br s, 2H, NH₂), 4.08 (q, ³J = 7.0 Hz, 2H), 3.31 (t, ³J = 6.2 Hz, 2H), 2.89 (m, 2H), 2.10 (d, ³J = 6.9 Hz, 2H), 1.87 (m, 2H), 1.76-1.63 (m, 7H), 1.56 (m, 1H), 1.48 (t, ³J = 7.0 Hz, 3H), 1.46 (m, 1H), 1.39-1.33 (m, 2H), 1.25-1.10 (m, 3H), 0.84 (m, 2H); ¹³C NMR (CDCl₃, 126 MHz) δ 164.4, 158.6, 150.4, 142.8, 114.0, 97.5, 73.7, 66.1, 64.8, 54.1 (2C), 45.3, 36.3, 35.3, 32.2 (2C), 30.1 (2C), 26.9, 26.3 (2C), 15.0; IR (neat, cm⁻¹) ν 3463, 3386, 3322, 3206, 2916, 2847, 2797, 2759, 1651, 1626, 1581, 1254, 1123, 815, 577; HRMS (ESI) *m/z* calcd. for C₂₂H₃₅IN₃O₂ [M+H]⁺ 500.1769, found 500.1768.

4-amino-*N*-{[1-(cyclohexylmethyl)-4-piperidyl]methyl}-2-(2-fluoroethoxy)-5-iodobenzamide (18d). The compound was prepared from 4-amino-*N*-{[1-(cyclohexylmethyl)-4-piperidyl]methyl}-2-(2-fluoroethoxy)benzamide (**17b**) (75 mg, 0.19 mmol) according to *procedure (m)* with a stirring at room temperature for 1 h. After a purification by flash chromatography on silica gel column (DCM/MeOH, gradient 100:0 to 90:10), the compound (**18d**) was obtained as a white solid (66 mg, 67% isolated yield); mp 162 °C; ¹H NMR (CDCl₃, 500 MHz) δ 8.46 (s, 1H), 7.70 (br s, 1H, NH), 6.23 (s, 1H), 4.79 (dt, ²J = 47.3 Hz, ³J = 4.1 Hz, 2H), 4.44 (br s, 2H, NH₂), 4.25 (dt, ³J = 27.2 Hz, ³J = 4.1 Hz, 2H), 3.31 (t, ³J = 6.3 Hz, 2H), 2.85 (m, 2H), 2.07 (d, ³J = 7.1 Hz, 2H), 1.83 (m, 2H), 1.75-1.63 (m, 7H), 1.55 (m, 1H), 1.46 (m, 1H), 1.32 (m, 2H), 1.25-1.10 (m, 3H), 0.85 (m, 2H); ¹³C NMR (CDCl₃, 126 MHz) δ 164.1, 157.8, 150.4, 143.1, 114.5, 97.7, 81.3 (d, ¹J = 172.4 Hz), 74.6, 68.0 (d, ²J = 19.3 Hz), 66.3, 54.2 (2C), 45.5, 36.3, 35.4, 32.2 (2C), 30.2 (2C), 27.0, 26.3 (2C); ¹⁹F NMR (CDCl₃, 476 MHz) δ -224.44 (tt, ²J = 48.1 Hz, ³J = 27.7 Hz); IR (neat, cm⁻¹) ν 3441, 3410, 3295, 3181, 2921, 2851, 1628, 1259, 1069, 891, 596; HRMS (ESI) *m/z* calcd. for C₂₂H₃₄FIN₃O₂ [M+H]⁺ 518.1674, found 518.1675.

4-amino-*N*-{[1-(cyclohexylmethyl)-4-piperidyl]methyl}-5-iodo-2-methoxybenzamide (18e). The compound was prepared from 4-amino-*N*-{[1-(cyclohexylmethyl)-4-piperidyl]methyl}-2-methoxybenzamide (**17c**) (200 mg, 0.56 mmol) according to *procedure (m)* with a stirring at room temperature for 2 h. After a purification by flash chromatography on silica gel column

(DCM/MeOH, gradient 100:0 to 90:10), the compound (**18e**) was obtained as a white solid (182 mg, 67% isolated yield); mp 174 °C; ¹H NMR (CDCl₃, 400 MHz) δ 8.44 (s, 1H), 7.69 (t, ³J = 5.5 Hz, 1H, NH), 6.27 (s, 1H), 4.44 (br s, 2H, NH₂), 3.88 (s, 3H), 3.30 (t, ³J = 6.2 Hz, 2H), 2.87 (m, 2H), 2.09 (d, ³J = 7.0 Hz, 2H), 1.85 (m, 2H), 1.76-1.62 (m, 7H), 1.57 (m, 1H), 1.47 (m, 1H), 1.34 (m, 2H), 1.27 (m, 3H), 0.85 (m, 2H); ¹³C NMR (CDCl₃, 100 MHz) δ 164.3, 159.2, 150.5, 142.9, 114.1, 96.8, 73.8, 66.1, 56.1, 54.2 (2C), 45.3, 36.4, 35.4, 32.2 (2C), 30.1 (2C), 26.9, 26.3 (2C); IR (neat, cm⁻¹) ν 3452, 3314, 3196, 2917, 2845, 1636, 1583, 1551, 1409, 1267, 1211; HRMS (EI) *m/z* calcd. for C₂₁H₃₂IN₃O₂ [M]⁺ 485.1540, found 485.1537.

4-amino-5-iodo-2-methoxy-*N*-[(1-propyl-4-piperidyl)methyl]benzamide (18f**).** The compound was prepared from 4-amino-2-methoxy-*N*-[(1-propyl-4-piperidyl)methyl]benzamide (**17d**) (50 mg, 0.16 mmol) according to *procedure (m)*. After a purification by chromatography on silica gel column (DCM/MeOH 90:10 to DCM/MeOH/Et₃N 90:10:2), the compound (**18f**) was obtained as a white solid (22 mg, 32% isolated yield); mp 114 °C; ¹H NMR (CDCl₃, 500 MHz) δ 8.41 (s, 1H), 7.79 (br s, 1H, NH), 6.29 (s, 1H), 4.47 (br s, 2H, NH₂), 3.90 (s, 3H), 3.33 (m, 4H), 2.68 (m, 2H), 2.43 (m, 2H), 1.96-1.76 (m, 7H), 0.95 (t, ³J = 7.4 Hz, 3H); ¹³C NMR (CDCl₃, 126 MHz) δ 164.7, 159.3, 150.8, 142.8, 113.5, 96.8, 73.7, 59.7, 56.2, 52.8 (2C), 44.5, 34.3, 27.8 (2C), 18.3, 11.6; IR (neat, cm⁻¹) ν 3395, 3197, 2959, 2933, 2649, 2531, 1623, 1587, 1538, 1257, 1213; HRMS (ESI) *m/z* calcd. for C₁₇H₂₇IN₃O₂ [M+H]⁺ 432.1150, found 432.1142.

5.2 Synthesis of radiotracers

Iodine-125 Radionuclide (specific activity ~629GBq/mg) was obtained from Perkinelmer.

[¹²⁵I]**18c** and [¹²⁵I]**18d** were obtained by incubation, for 30 min at room temperature, of a mixture containing 5 μ l of precursor (10 μ g/ μ l in ethanol), 3 μ l of glacial acetic acid, 3 μ l of 30% H₂O₂ and 10 mCi of carrier-free ¹²⁵I sodium iodide in 0.1 M aqueous NaOH. Radiotracers were isolated by a linear gradient HPLC run (from 5% acetonitrile, ACN, to 95% ACN, 10mM aqueous H₃PO₄, in 10 min).

HPLC was equipped with a reverse-phase column (Phenomenex Bonclone C18, Phenomenex, Schlieren, Switzerland) and radiotracers were eluted at a flow of 3 mL/min. Fractions containing radiotracers were diluted in water and loaded on a Sep-Pak cartridge (Sep-Pak C18, Waters,

Switzerland). Radiotracers were eluted with 0.5 ml of 95% ACN, 10mM H₃PO₄ and concentrated using a rotary evaporator, and the final products were diluted in saline prior to animal administration.

During the HPLC run, ultraviolet absorbance and radioactivity were monitored, allowing for the measurement of specific activity thanks to calibration curves established with the cold reference compound. Radiochemical yields were 47% and 61% for [¹²⁵I]**18c** and [¹²⁵I]**18d**, respectively. Specific activity of radiotracers was superior to 200 GBq/μmol.

5.3 *In vivo* and *Ex vivo* imaging experiments

Three Sprague Dawley Mdr1a KO (SD-Abcb1^{tm1sage}, Sigma Advance Genetic Engineering Labs, Boyertown, PA) rats (390 ± 22 g) were used to determine the efficacy of [¹²⁵I]**18c** (n=1) and [¹²⁵I]**18d** (n=2) to image 5HT₄R *in vivo*. Anesthetized animals (4% isoflurane for induction, 2.5% for maintenance) were placed in the USPECT with their head positioned in the center of the field of view. Body temperature was maintained at 37 ± 1 °C using a thermostatically controlled heating blanket. A 60 min acquisition was initiated upon a tail vein injection of tracer (26 ± 2.4 MBq). Immediately after the end of the acquisition, rats were euthanized by decapitation. The brains were quickly removed and frozen in pre-cold isopentane. Transverse sections (20 μm) were cut on a cryostat and exposed to phosphor imaging plates overnight (Fuji Photo Film Co., Tokyo, Japan). All experimental procedures were performed in accordance with the Swiss Federal Law on animal care under a protocol approved by the Ethical Committee on Animal Experimentation of the Canton of Geneva, Switzerland.

5.4 *In vitro* competition

Transverse sections (20 μm), from two Sprague Dawley Mdr1a KO rats, at the level of olfactory tubercles were cut on a cryostat. Slides were air-dried and stored at -20 °C. Sections were pre-incubated in 50mM Tris-HCl buffer (pH=7.4) for 15 min. Sections were then treated with ¹²⁵I-SB207710 (0.24 MBq/ml) in 50mM Tris-HCl buffer (pH=7.4) for 90 min in the presence of increased **18c** or **18d** concentrations (from 0.01 to 10 μM) or in the presence of 1 μM of SB207710. Sections were washed 3 times in ice cold buffer, dipped in ice-cold deionized water,

and air-dried. Finally, sections were exposed to phosphor imaging plates overnight (Fuji Photo Film Co., Tokyo, Japan).

5.2. Biological Methods

5.2.1 5-HT₄ binding experiment

For radioligand binding studies, 2.5 µg of proteins (5-HT₄B membrane preparations, HTS110M, Millipore) were incubated in duplicate at 25 °C for 60 min in the absence or the presence of 10⁻⁶ or 10⁻⁸M of each drug and 1 nM [³H]-GR 113808 (VT 240, ViTrax) in 25 mM Tris buffer (pH 7.4). At the end of the incubation, homogenates were filtered through Whatman GF/C filters (Alpha Biotech) presoaked with 0.5% polyethylenimine using a Brandel cell harvester. Filters were subsequently washed three times with 4 mL of ice-cold 25 mM Tris buffer (pH 7.4). Non specific binding was evaluated in parallel in the presence of 30 µM serotonin.

The method was validated from saturation studies: six concentrations of [³H]GR113808 were used to give final concentrations of 0.0625–2 nM, and nonspecific binding of [³H]GR113808 was defined in the presence of 30 µM serotonin to determine the K_d and the B_{max}. For competition studies, [³H]GR113808 was used to give a final concentration of 0.2 nM.

Percentages of inhibition of the binding of [³H]GR 113808 were obtained for concentrations of 10⁻⁶ and 10⁻⁸ M of the ligands tested. For some of these compounds, affinity constants were calculated from five-point inhibition curves using the EBDA-Ligand software and expressed as K_i ± SD.

5.2.2 Lipophilicity evaluation

Log P estimation was carried out using an isocratic liquid chromatography method for basic compounds derived from method described by Henchoz [29].

All experiments were performed on a UHPLC Agilent 1290 Infinity system (Agilent Technologies, Santa Clara, California, USA) equipped with a PDA detector 1260 operating at 220, 240, 254, 290 and 350 nm for all compounds. The chromatographic system was controlled by Open LAB CDS LC Chemstation™ software (revision C01.05). Retention time measurement was performed at 27 °C, with a flow rate 0.6 mL.min⁻¹, and by using an Acquity BEH Shield RP18 column (1.7 µm, 2.1 x 50 mm) from Waters (Milford, MA, USA). Mobile phase was

composed by various percentage of methanol as organic modifier (35-85 %) and an aqueous basic buffer, a triethylammonium acetate solution at pH 11.5 adjusted by sodium hydroxide addition, in order to keep calibration and newly synthesized basic compounds in their neutral form.

Briefly, Log P was estimated by plotting the known log P values of 13 basic standard compounds with their log $k_{49\%}$, which is the retention factor at 49% methanol. Calculation of log $k_{49\%}$ values was performed from retention time (t_R) measurement at three different mobile phase compositions. Each compound was injected (1 μ L) once with each mobile phase composition and t_R determined from the apex of the peak. Finally, log $k_{49\%}$ values were obtained by extrapolation to 49 % organic modifier using linear relationships between log k values and methanol percentage ($r^2 > 0.99$ for all compounds).

Calibration curve was built with Log P_{oct} values of standards obtained from literature versus their calculated Log $K_{49\%}$ which is the logarithm of retention factor K at 49 % methanol in mobile phase. Linear relationships between Log P_{oct} and calculated log $K_{49\%}$ was expressed by the following equation :

$$\text{Log } K_{49\%} = 0.5226 \times \text{Log } P_{oct} - 0.7058 \quad \text{with } r^2 = 0.978 \quad \text{and } F = 444$$

Calculated logP were obtained using MarvinSketch 5.2.6 (<http://www.chemaxon.com/products/marvin/marvinsketch>/<http://www.chemaxon.com/products/marvin/marvinsketch/>) or Molinspiration (<http://www.molinspiration.com/cgi-bin/properties>).

5.2.3. Parallel Artificial Membrane Permeability Assay (PAMPA).

The PAMPA-BBB experiment were conducted using the Pampa Explorer Kit (Pion Inc) according to manufacturer's protocol. Each stock compound solution (20 mM in DMSO) were diluted in Prisma HT buffer pH 7.4 (pION) to 100 μ M. 200 μ L of this solution (n=6) was added to donor plate (P/N 110243). 5 μ L of the BBB-1 Lipid (P/N 110672) was used to coat the membrane filter of the acceptor plate (P/N 110243). 200 μ L of the Brain Sink Buffer (P/N 110674) was added to each well of the acceptor plate. The sandwiches were incubated at room temperature for 4 h, without stirring. After incubation, the UV-visible spectra were measured with the microplate reader (Tecan infinite M200) and the $-\log P_e$ were calculated for each compound by using the PAMPA Explorer software v. 3.7 (pION). Quality control standards with

known $-\log Pe$ values were used as references: the highly permeable corticosterone ($-\log Pe = 4.6$) and the low permeable theophylline ($-\log Pe < 6.0$) for the PAMPA-BBB experiments, and the low/moderately permeable ketoprofen and antipyrine ($-\log Pe = 5.8$ at pH 7.4) for the PAMPA-GIT assays.

Acknowledgments

This work was supported by funding from the French Agence Nationale de la Recherche Project MALAD ANR-12-JS007-0012-01 and the Interreg program AI-Chem Channel. This work was also supported by the Swiss National Science Foundation (no. 310030-120369). The authors are grateful for the contribution of the “Association IFRAD Suisse”, which was created in 2009 at the initiative of the “Fondation pour la Recherche sur Alzheimer” (formerly IFRAD France).

Abbreviations

AD Alzheimer's Disease

BBB blood brain barrier

5-HT 5-Hydroxytryptamine, serotonin

Ce cerebellum

CNS central nervous system

Cx cortex

Hip hippocampus

Ip interpeduncular nucleus

PET positron emission tomography

SPECT single photon emission computed tomography

St striatum

Thal thalamus

Tu olfactory tubercles

Supplementary information

Supplementary data related to this article can be found at

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List of Captions

Figure 1. A: Structures of reference 5-HT₄R ligands; **B:** 5-HT₄R PET and SPECT radiotracers; **C:** 5-HT_{2A}R SPECT radiotracer; **D:** targeted structures.

Scheme 1. Synthetic route of aryl ketone derivatives 14a-b^a

^aReagents, conditions and yields: (a) H₂SO₄ cc., MeOH, 16h, reflux, 90%; (b) iodoethane, K₂CO₃, DMF, 70°C, overnight, 61%; (c) 2-fluoroethyl 4-methylbenzenesulfonate, K₂CO₃, DMF, 110°C, 2h, 62%; (d) 1N NaOH aq., EtOH, rt, overnight, 94-96%; (e) CDI, dry THF, rt, 15h, then potassium 3-ethoxy-3-oxopropanoate, MgCl₂, 40°C, 24h, 23-36%; (f) *tert*-butyl 4-(iodomethyl)piperidine-1-carboxylate, K₂CO₃, DMF, rt, 48h; (g) KOH, EtOH/H₂O (5:1), reflux, 5h, 70-74% over two steps; (h) TFA, DCM, rt, 1h; (i) bromomethylcyclohexane, K₂CO₃, 110°C, 5h, 43-58% over two steps.

Scheme 2. Synthetic route of benzamide derivatives 17a-d^b

^bReagents, conditions and yields: (j) alkyl-halogenated derivatives, K₂CO₃, EtOH, reflux, 15-24h, 92-93%; (k) LiAlH₄, dry THF, addition 0°C then rt, 1-2h, reflux, 3h, 71-96%; (l) EDCI, HOBt, Et₃N, DMF, rt, 18-72h, 17-44%.

Scheme 3. Synthesis of iodinated compounds 18a-f^c and ¹²⁵I radiolabeling of compounds 17a-b^c

^cReagents, conditions and yields: (m) NaI, acetic acid/30% H₂O₂ 2:1, rt, 1-2h, 18-71%; (n) Na¹²⁵I, acetic acid/30% H₂O₂ 2:1, rt, 30 min, 47-61%.

Table 1. 5-HT₄R affinity for the compounds **14a-b**, **17a-d** and **18a-f**; calculated and measured lipophilicities and permeabilities in the PAMPA-BBB assay with their predictive penetration in the CNS for the compounds **18a-f**.

Figure 2. *In vivo* SPECT imaging showing activity distribution in the brain after injection of [¹²⁵I]**18c** or [¹²⁵I]**18d**. Representative MRI images in coronal (A), sagittal (B) and axial (C) planes. Arrows indicate areas of interest: olfactory tubercles (yellow), Caudate-putamen (white) and cerebellum (green). Images of SPECT scan between 10 and 60 minutes after injection of [¹²⁵I]**18c** (D-F) or [¹²⁵I]**18d** (G-I) coregistered with MRI template.

Figure 3. *Ex vivo* autoradiograms in the brain after injection of [¹²⁵I]**18c** or [¹²⁵I]**18d**. Anatomic atlas templates adapted from the Paxinos and Watson atlas (A), autoradiograms obtained 60 minutes after injection of [¹²⁵I]**18c** (B) or [¹²⁵I]**18d** (C). Cx: cortex, Ce: cerebellum, Hip: hippocampus, Ip: interpeduncular nucleus, Tu: olfactory tubercles, St: striatum, Thal: thalamus.

Figure 4. *In vitro* competition between [¹²⁵I]**1** and **18c**, **18d** or **1**. Localisation of olfactory tubercles (Tu) is shown in a coronal slice, adapted from the Paxinos and Watson atlas (A). Representative autoradiograms obtained by incubation of [¹²⁵I]**1** in the presence of growing concentrations (0, 0.01, 0.1, 1, 10 μM, from B to F, respectively) of **18c** (topline) or **18d** (line below) or in the presence of 1 μM of **1** (G).

Table 1. 5-HT₄R affinity for the compounds **14a-b**, **17a-d** and **18a-f**; calculated and measured lipophilicities and permeabilities in the PAMPA-BBB assay with their predictive penetration in the CNS for the compounds **18a-f**.

Compound	Y	R ¹	R ²	K _i 5-HT ₄ R (nM) %Inhibition 10 ⁻⁶ M/10 ⁻⁸ M	Lipophilicity		Permeability	
					<i>cLogP</i> ^a - <i>cLogP</i> ^b	<i>LogP</i> exp.	<i>Pe</i> (10 ⁻⁶ cm.s ⁻¹)	Prediction
14a	CH ₂	C ₂ H ₅		24.7 ± 5.3 (n=3) ^d 100% / 38%	n.d.	n.d.	n.d.	n.d.
18a	CH ₂	C ₂ H ₅		86 ± 19.4 (n=3) ^d 100% / 65%	5.06 - 6.13	6.4 ± 0.5	8.08	CNS +/-
14b	CH ₂	C ₂ H ₄ F		39.9 ± 5.3 (n=3) ^d 100% / 27%	n.d.	n.d.	n.d.	n.d.
18b	CH ₂	C ₂ H ₄ F		521 ± 261 (n=3) ^d 100% / 22%	4.91 - 6.05	5.6 ± 0.4	10.36	CNS +/-
17a	NH	C ₂ H ₅		6.9 ± 0.6 (n=3) ^d 100% / 51%	n.d.	n.d.	n.d.	n.d.
18c	NH	C ₂ H ₅		8.64 ± 0.37 (n=3) ^d 100% / 20%	3.95 - 5.63	4.9 ± 0.5	24.38	CNS +
17b	NH	C ₂ H ₄ F		8.1 ± 3.4 (n=3) ^d 100% / 38%	n.d.	n.d.	n.d.	n.d.
18d	NH	C ₂ H ₄ F		14.7 ± 2.9 (n=3) ^d 100% / 30%	3.79 - 5.55	5.2 ± 0.4	32.29	CNS +
17c	NH	CH ₃		n.d. 72% / 40% ^g	n.d.	n.d.	n.d.	n.d.
18e	NH	CH ₃		13.9 ± 3.7 (n = 3) ^c 100% / 29%	3.59 - 5.25	5.0 ± 0.4	31.60	CNS +
17d	NH	CH ₃		n.d. 79% / 5% ^g	n.d.	n.d.	n.d.	n.d.

18f	NH	CH ₃		68.7 ± 11.4 (n=3) ^c 96% / 11%	2.36 - 3.84	3.5 ± 0.4	22.68	CNS +
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n.d. not determined / ^a calculated with MarvinSketch / ^b calculated with Molinspiration / ^c guinea pig receptors / ^d human receptors

A **C** **B**

[¹²³I]**1** ([¹²³I]-SB207710), X=¹²³I, R₁=*n*-Bu
 [¹¹C]**2** ([¹¹C]-SB207145, X=Cl), R₁=¹¹CH₃
 [¹⁸F]**3** ([¹⁸F]-MNI-698, X=Cl), R₁=C₂H₄¹⁸F
 [¹⁸F]**4** ([¹⁸F]-MNI-699, X=Cl), R₁=C₃H₇¹⁸F

[¹¹C]**5** ([¹¹C]-RX-1), R₂=¹¹CH₃
 [¹⁸F]**6** ([¹⁸F]-RX-2), R₂=CD₂¹⁸F

[¹²⁵I]**7**, X=CH
 [¹²⁵I]**8**, X=N

D

A

B

C

D

E

F

G

18a, X= I, Y = CH₂, R¹ = C₂H₅, R² = C₇H₁₃, 18%

18b, X= I, Y = CH₂, R¹ = C₂H₄F, R² = C₇H₁₃, 41%

18c, X= I, Y = NH, R¹ = C₂H₅, R² = C₇H₁₃, 71%

18d, X= I, Y = NH, R¹ = C₂H₄F, R² = C₇H₁₃, 67%

18e, X= I, Y = NH, R¹ = CH₃, R² = C₇H₁₃, 67%

18f, X= I, Y = NH, R¹ = CH₃, R² = C₃H₇, 32%

[¹²⁵I]**18c**, X= ¹²⁵I, Y = NH, R¹ = C₂H₅, R² = C₇H₁₃, 47%

[¹²⁵I]**18d**, X= ¹²⁵I, Y = NH, R¹ = C₂H₄F, R² = C₇H₁₃, 61%

Synthesis and evaluation of novel serotonin 4 receptor radiotracers for single photon emission computed tomography

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