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Radiosynthesis, in vitro and in vivo evaluation of ^{123}I-labeled anandamide analogues for mapping brain FAAH

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ABSTRACT

Fatty acid amide hydrolase (FAAH) is one of the main enzymes responsible for terminating the signaling of endocannabinoids, including anandamide. This paper is the first report of the synthesis, [^{123}I]-labeling and in vitro and in vivo evaluation of anandamide analogues as potential metabolic trapping radioligands for in vivo evaluation of brain FAAH. *N*-(2-Iodoethyl)linoleoylamide (**2**) and *N*-(2-iodoethyl)arachidonylamide (**4**) were synthesized with good yields (75% and 86%, respectively) in a two steps procedure starting from their respective acids. In vitro analyses, performed using recombinant rat FAAH and [^{3}H]-anandamide, demonstrated interaction of **2** and **4** with FAAH (IC_{50} values of 5.78 μM and 3.14 μM, respectively). [^{123}I]-**2** and [^{123}I]-**4** were synthesized with radiochemical yields of 21% and 12%, respectively, and radiochemical purities were >90%. Biodistribution studies in mice demonstrated brain uptake for both tracers (maximum values of 1.23%ID/g at 3 min pi for [^{123}I]-**2** and 0.58%ID/g at 10 min pi for [^{123}I]-**4**). However, stability studies demonstrated the sensitivity of both tracers to dehalogenation.

1. Introduction

Since the discovery in the early 1990s of specific membrane receptors of marijuana's (*Cannabis sativa*) psychoactive compound Δ^9-tetrahydrocannabinol (Δ^9-THC) and the revelation of a whole endogenous signaling system known as the endocannabinoid system, considerable research has been devoted to the characterization of the different members of this endocannabinoid system. Besides the cannabinoid receptors CB_1 and CB_2, the endocannabinoid system also comprises their endogenous ligands (the endocannabinoids, with anandamide (*N*-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) being the most studied ones) and the proteins for their synthesis and inactivation.[1]

Fatty acid amide hydrolase (FAAH) is the main enzyme responsible for terminating the signaling of AEA by hydrolysis of the amide bond after uptake in the cell by the still controversial AEA membrane transporter (AMT).[2,3] (For reviews on AMT see Refs. 4,5). FAAH is a dimeric integral membrane enzyme, found predominantly in microsomal and mitochondrial fractions and is the first characterized mammalian member of the amidase signature family.[6–8] It is a nonclassical serine hydrolase that utilizes a Ser-Ser-Lys catalytic triad instead of the more common Ser-His-Asp catalytic triad with serine-241 as the catalytic nucleophile in the active site of the enzyme.[8–10] FAAH may act as a hydrolytic enzyme not only for anandamide but also for the anti-inflammatory compound *N*-palmitoylethanolamine (PEA),[11] the sleep inducing lipid *cis*-9-octadecenoamide (oleamide),[12] and even esters such as 2-AG (Fig. 1).[13]

The importance of FAAH as a regulatory enzyme for key physiological functions is suggested by studies with transgenic mice lacking FAAH. They possess high endogenous concentrations of anandamide and related fatty acid amides in the brain that correlate with CB_1 dependent behavioral responses including hypomotility, analgesia, catalepsy and hypothermia.[14] An increasing number of reports suggests that the symptoms of several neurological and neuropsychiatric disorders could be caused by changes in the endocannabinoid biosynthesis and degradation, including: addiction,[15–18] schizophrenia,[19,20] anxiety,[21] depression,[22,23] multi-

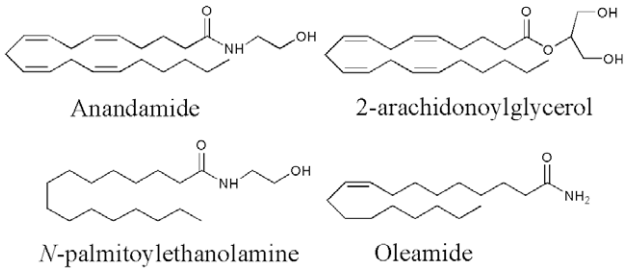

Figure 1. Chemical structures of representative FAAH substrates.

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ple sclerosis,[24,25] epilepsy,[26] Parkinson's disease,[27,28] and Huntington's disease.[29]

To better understand the relationship between the FAAH-endocannabinoid system and neuronal and neuropsychiatric disorders and to find cause-effect relationships between changes in the expression/activity of FAAH and pathological conditions, in vivo molecular imaging using metabolic trapping can be helpful. The principle of this method is that a radiolabeled substrate analogue passes the blood–brain-barrier (BBB), is taken up in the cell and hydrolyzed by FAAH to produce a labeled amine which has limited permeability through the BBB and becomes trapped in neuronal cells. In that manner, we can visualize the enzyme and quantify the amount and functionality of FAAH. Metabolic trapping has already been successfully applied for evaluation of acetylcholinesterase activity in the brain for Alzheimer's disease diagnosis using [^{18}F]-, [^{11}C]- and [^{123}I]-labeled acetylcholine analogues.[30–33] Other well-known examples are the use of the glucose analogue [^{18}F]-2-fluorodeoxyglucose ([^{18}F]-FDG) to assess rates of cerebral glucose utilization for detection of brain tumours, epileptic foci and Alzheimer's disease[34,35] and the use of [^{99m}Tc]-ethylene cysteine dimer ([^{99m}Tc]-ECD) for the study of regional cerebral perfusion.[36]

The visualization of FAAH in vivo with positron emission tomography (PET) or single photon emission tomography (SPECT) forms a powerful method for the study of several neuropsychiatric diseases.

Since no tracers for in vivo evaluation of FAAH currently exist, the purpose of this study was to synthesize and evaluate ligands based on an anandamide template with a view to discovering metabolic trapping ligands for visualization of this enzyme in the brain using SPECT. Structure–activity relationships studies demonstrated that anandamide analogues with an electronegative head group substituent are better FAAH substrates than their unsubstituted counterparts[37]; therefore, we chose to develop an anandamide analogue based [^{123}I]-SPECT ligand for visualization of FAAH. Hereby, we describe the synthesis of *N*-(2-iodoethyl)linoleoylamide (**2**) and *N*-(2-iodoethyl)arachidonylamide (**4**). Both **2** and **4** were tested in vitro using recombinant rat FAAH for evaluation of their interaction with the enzyme. Subsequently, the ^{123}I-derivatives [^{123}I]-**2** and [^{123}I]-**4** were prepared for biodistribution studies using NMRI mice to evaluate their potentials as radioligand for mapping brain FAAH in vivo.

2. Results and discussion

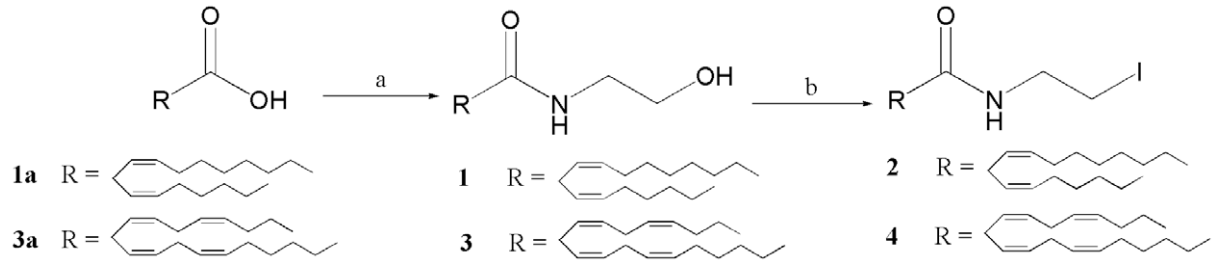
2.1. Chemistry

The most often described method for the synthesis of anandamide analogues is by preparation of the acyl chloride followed by treatment with the appropriate amine or alcohol.[38] However, because of the instability of linoleoyl and arachidonyl chloride, using this method did not result in *N*-(2-hydroxyethyl)linoleoylamide (**1**) and arachidonylethanolamide (**3**) in a high yield procedure. Another, although less common, synthetic route is the direct condensation between in situ pre-activated arachidonic acid or linoleic acid, employing the coupling agent carbonyldiimidazole (CDI), and the corresponding amine, thus allowing for a one-pot amide formation. Using this method, we were able to synthesize **1** and **3** in high yields (78% and 64%, respectively). In practice, the imidazolides of arachidonic acid and linoleic acid were preformed for 1 h and then ethanolamine was added to form the corresponding acylethanolamide. Attempts to synthesize *N*-(2-iodoethyl)linoleoylamide (**2**) and *N*-(2-iodethyl)arachidonylamide (**4**) by reaction of the activated acid with 2-iodoethylamine did not give the compounds in satisfactory purity nor high yield. The 2-iodoethylamine was obtained by deprotection of *N*-trityl-2-iodoethanamine with 1-hydroxybenzotriazole (HOBt) in trifluoroethanol or obtained after deprotection of *tert*-butyl-2-iodoethyl-carbamate with HCl (4 M in dioxane) or trifluoroacetic acid. On the other hand, conversion of compounds **1** and **3** to the iodide by treatment with triphenylphospine/I_2 did result in compounds **2** and **4**, respectively, in acceptable purity and high yields (75% and 86%, respectively) (Scheme 1).

2.2. In vitro evaluation

2.2.1. FAAH assay

A prerequisite for a metabolic trapping ligand is that the compound is a substrate of the enzyme under investigation. For the anandamide analogues synthesized here, it is not known whether they are substrates for FAAH. The FAAH assay used in this study allows us to investigate the interaction between recombinant rat FAAH (rFAAH) and compounds **2** and **4** by their ability to prevent the enzyme from hydrolyzing [^{3}H]-AEA. It is important to emphasize that using this approach no information is given on the efficacy of the compounds as substrates but rather allows for determination of their affinity for the enzyme.[39] Experiments were performed using ten concentrations of test compound **2** or **4** in the range of 5 μM–1.5 nM. Potencies of **2** and **4** to inhibit the hydrolysis of [^{3}H]-AEA by FAAH are expressed as pIC_{50}. Compound **1** is known to be a substrate for FAAH. Compared to AEA (rate = 100%), **1** is hydrolyzed to an extent of about 75%.[37] As a result of this observation, we used **1** as a reference compound as it inhibits [^{3}H]-AEA metabolism by acting as a competing substrate. The results are presented in Figure 2. A pIC_{50} value of 5.12 ± 0.05 (corresponding to an IC_{50} value of 7.58 μM) was obtained for reference compound **1**, thus demonstrating a comparable potency to the endogenous FAAH substrate palmitoylethanolamide (PEA, pIC_{50} value of 5.30) reported by Vandevoorde et al.[40] Compounds **2** and **4** gave an IC_{50} value of 5.78 ± 0.69 μM and 3.14 ± 0.30 μM, respectively. These results are in line with SAR studies showing that fatty acid ethanolamides containing fewer than four *cis* nonconjugated double bonds are not as good substrates as AEA.[37,41]



Scheme 1. Reagents and conditions: (a) CDI, CH_2Cl_2, room temperature, 1 h, then $NH_2CH_2CH_2OH$, room temperature, 3 h (yield: compound **1** 78%, compound **3** 64%). (b) PPh_3, I_2, imidazole, CH_2Cl_2, room temperature, 3 h (yield: compound **2** 75%, compound **4** 86%).

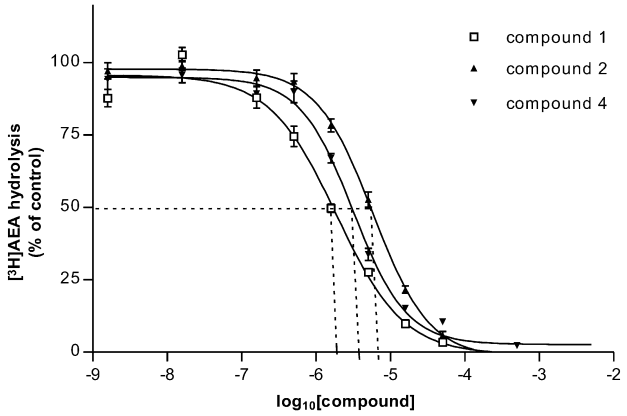


Figure 2. Inhibition of the hydrolysis by recombinant rat FAAH of 2 μM [^{3}H]-AEA by compound **1**, **2** and **4**. Shown are means of three experiments, with no preincubation of the compounds with rFAAH prior to addition of [^{3}H]-AEA. pIC_{50} values are shown as a dotted line on the figure.

The results are comparable with the IC_{50} value of FAAH substrate PEA and give an indication that compounds **2** and **4** show an interaction with FAAH, possibly as substrates.

One way of distinguishing inhibitors from competing substrates is to preincubate the compounds with rFAAH prior to addition of [^{3}H]-AEA: for a competing substrate, the preincubation is expected to reduce the observed potency of the compound.[42] Therefore, we selected the concentrations of test compounds **2** or **4** that gave 20%, 50% and 80% inhibition of [^{3}H]-AEA metabolism in the previously described experiment and preincubated them with rFAAH for 0, 15, 30 or 60 min before adding [^{3}H]-AEA. The preincubation showed little effect on the pIC_{50} values of compound **2** (calculated IC_{50} values after 0, 15, 30 and 60 min preincubation with rFAAH: 6.20 μM, 6.66 μM, 7.64 μM, 7.33 μM, respectively); however, compound **4** showed a larger decrease in potency with preincubation. Calculated IC_{50} values for compound **4** of 4.311 μM, 7.93 μM, 9.89 μM, 10.07 μM (0, 15, 30 and 60 min preincubation, respectively) suggest metabolization of **4** by rFAAH which results in a decrease of potency to inhibit [^{3}H]-AEA metabolism.

From the above experiments it appears that both **2** and **4** show an interaction with rFAAH. The preincubation experiments demonstrated that **4** interacts with FAAH as a competing substrate while compound **2** probably binds to FAAH without being efficiently metabolized.

2.2.2. HPLC analysis of metabolization

To further identify **2** and **4** as substrates for FAAH, a HPLC method was used to demonstrate the generation of linoleic acid or arachidonic acid as a result of enzymatic metabolization after incubation of the compounds with FAAH. An experiment with the known FAAH substrate AEA was performed to validate the method. Since AEA is hydrolyzed by FAAH to the fatty acid arachidonic acid and ethanolamine, HPLC analysis with UV detection of test solution containing AEA and rFAAH reveals the generation of arachidonic acid. Incubation of **2** and **4** with rFAAH resulted in the formation of linoleic acid and arachidonic acid, respectively. No formation of fatty acid could be detected in the blank experiments, containing assay buffer instead of rFAAH. To rule out spontaneous formation of fatty acid during incubation, the stability of anandamide, **2** and **4** in assay buffer was studied by incubation at 37 °C. Hundred microliters of the test solution was injected into the HPLC system after the first minute and at 15 min intervals for 1 h. The results showed that no formation of fatty acid was detected during this time.

Methyl arachidonoyl fluorophosphonate (MAFP) is a potent irreversible inhibitor of FAAH. Preincubation of FAAH with MAFP blocks the enzymatic activity, resulting in no formation of fatty acid in case the compounds are genuine FAAH substrates. rFAAH was preincubated with 10 nM of MAFP in assay buffer for 10 min at 37 °C followed by addition of AEA, **2** or **4** and another 20 min incubation at 37 °C. HPLC analysis revealed no formation of fatty acid, indicating inhibition of conversion of AEA, **2** and **4** by FAAH with MAFP preincubation.

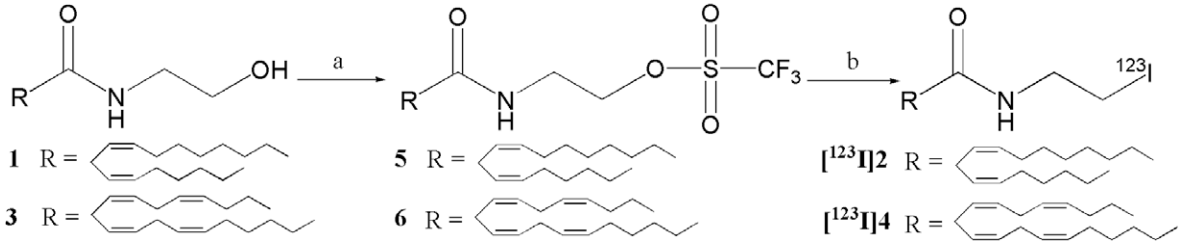
Although the above described FAAH assay did us assume that only **4** interacts with FAAH as a substrate while **2** interacts with FAAH without being efficiently metabolized, these data support the identity of both **2** and **4** as FAAH substrates.

2.3. Radiochemistry

Radiochemical synthesis of [^{123}I]-**2** and [^{123}I]-**4** was conducted by a nucleophilic substitution of the triflate precursor using n.c.a. (no carrier added) [^{123}I]NaI (Scheme 2). Briefly, each time fresh 2-(linoleoylamido)-ethyl trifluoromethanesulfonate (**5**) or 2-(arachidonylamido)-ethyl trifluoromethanesulfonate (**6**) was prepared and used without further purification. The triflate precursor (4 mg) was dissolved in dry acetone and left to react with [^{123}I]NaI for 30 min at elevated temperature (50 °C for [^{123}I]-**2** and 40 °C for [^{123}I]-**4**). [^{123}I]-**2** and [^{123}I]-**4** were separated from radioactive impurities by analytical HPLC and formulated in a 0.9% NaCl solution containing ethanol (8%) for in vivo evaluation. Radiochemical yields were 21 ± 3% (n = 6) for [^{123}I]-**2** and 12 ± 6.3% (n = 4) for [^{123}I]-**4**. Identification of the collected tracer was performed by comparing retention times on HPLC between the radioactive labeled product [^{123}I]-**2** and [^{123}I]-**4** and the unlabeled iodinated molecules **2** and **4**. Co-elution confirmed the identity of [^{123}I]-**2** and [^{123}I]-**4**. HPLC analysis showed a chemical and radiochemical purity of >90%.

2.4. Uptake of [^{123}I]-2 and [^{123}I]-4 in mouse brain

To investigate the uptake of [^{123}I]-**2** and [^{123}I]-**4** in the brain, a general biodistribution in mice was conducted. Adult male NMRI were injected iv in the tail vein with 4–5 μCi of [^{123}I]-**2** or [^{123}I]-**4**. The radioactivity concentrations of various tissues as a function



Scheme 2. Reagents and conditions: (a) Tf_2O, TEA, CH_2Cl_2, −20 to 0 °C, 1h (yield: compound **5** 72%, compound **6** 88%). (b) [^{123}I]NaI, acetone, 50 °C ([^{123}I]-**2**), 40 °C ([^{123}I]-**4**), 30 min.

of time following administration of [^{123}I]-**2** or [^{123}I]-**4** are shown in Figure 4.

Both [^{123}I]-**2** and [^{123}I]-**4** demonstrate uptake in brain, with the highest uptake at 3 min for [^{123}I]-**2** (1.23%ID/g) and at 10 min for [^{123}I]-**4** (0.579%ID/g), followed by a continuous decrease until the end of the study (Fig. 3). The uptake in the intestines remained rather constant and low throughout the study (1.84%ID/g and 1.33%ID/g in small intestines and large intestines, respectively, at 10 min pi for [^{123}I]-**2** and 1.46%ID/g and 1.04%ID/g in small intestines and large intestines, respectively, at 10 min pi for [^{123}I]-**4**), while the uptake in bladder/urine continuously increased over time, indicating that renal elimination is the primary excretory pathway. Also, an increase in uptake of radioactivity was observed in stomach (from 2.58%ID/g at 1 min pi to 24.57%ID/g at 1 h for [^{123}I]-**2** and from 3.10%ID/g at 1 min to 17.74%ID/g at 1 h for [^{123}I]-**4**). Since the transport from iodide from the bloodstream into the gastric lumen is a known phenomenon[44], the high uptake in stomach indicates dehalogenation of [^{123}I]-**2** and [^{123}I]-**4**.

2.5. Tracer dehalogenation

Compounds containing an iodine atom in aliphatic position are generally not very stable due to the relatively poor biochemical stability of the C-I bond.[44] Nevertheless, a relatively stable methylene-iodine bond was found in vivo with iodoethylamides.[45] Since our in vivo results lead us to suspect in vivo dehalogenation of [^{123}I]-**2** and [^{123}I]-**4**, tracer stability studies were performed using HPLC and thin-layer chromatographic (TLC) analysis with radioactivity detection. TLC analysis of the radioligands directly after HPLC purification and concentration demonstrated an acceptable stability of the tracer with 95% authentic [^{123}I]-**2** and 90% authentic [^{123}I]-**4**. An ex vivo stability experiment in mouse blood and brain tissue was performed. Radioactivity was extracted with acetonitrile from the plasma and brain of mice and submitted to HPLC and TLC analysis. From blank samples, spiked with [^{123}I]-**2**, an extraction efficiency of 94 ± 3.5% (n = 3) for brain tissue and 94 ± 1.5% (n = 3) for plasma was obtained. For [^{123}I]-**4**, extraction efficiencies of 89 ± 1.2% (n = 3) for brain tissue and 92 ± 0.6% (n = 3) were obtained. However, TLC and HPLC analysis of the supernatant revealed that, in the case of [^{123}I]-**2**, >45% of the extracted radioactivity was present in the form of free [^{123}I]I$^-$, and even >60% of free [^{123}I]I$^-$ in the case of [^{123}I]-**4**. Determination of authentic [^{123}I]-**2** in plasma and brain tissue 3 min postinjection, demonstrated almost complete metabolization of the labeled compound. Besides a small fraction of authentic [^{123}I]-**2** (<2%) in plasma, only free [^{123}I]I$^-$ could be observed in brain tissue.

Since these experiments proved the sensitivity of the radioligands for rapid dehalogenation, they will not be of any practical

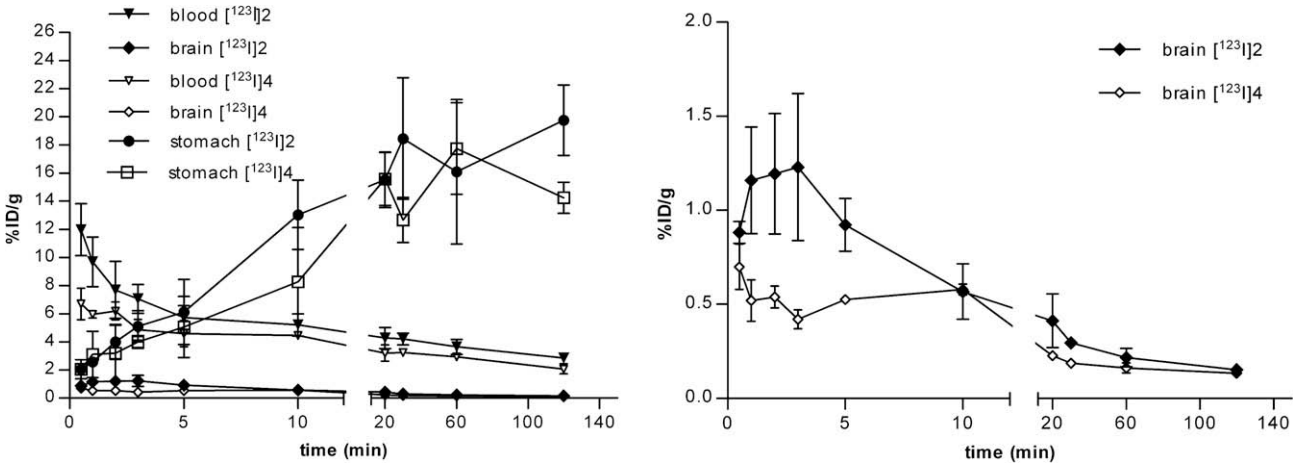


Figure 3. Time dependency of uptake (%ID/g) of [^{123}I] radioactivity in blood, brain and stomach after iv injection of [^{123}I]-**2** or [^{123}I]-**4** in mice.

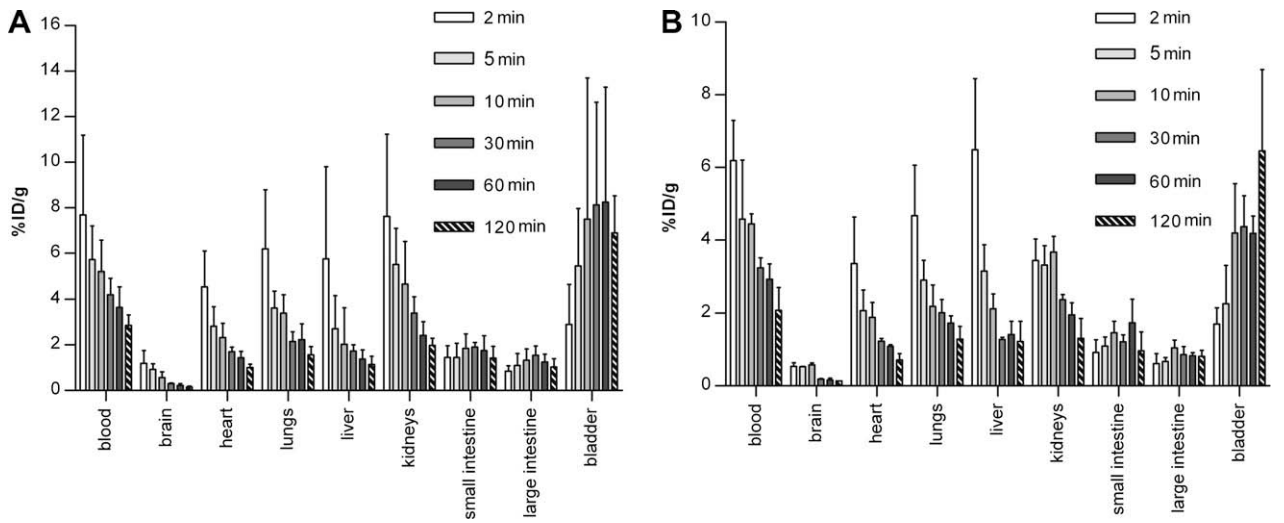


Figure 4. Uptake (%ID/g) of radioactivity in mice organs at various time points after iv injection of [^{123}I]-**2** (A) or [^{123}I]-**4** (B).

use for the in vivo visualization of FAAH despite their good in vitro results. However, since the interest of FAAH in normal and diseased states, the search for radioligands for visualization of the enzyme should be continued. Further research is necessary to develop FAAH radioligands for fast and objective diagnosis of mental illnesses like depression and schizophrenia, as well as for evaluation of usefulness of newly developed FAAH inhibitors. Iodinated endocannabinoid analogues less sensitive to dehalogenation as well as [^{18}F]- or [^{11}C]-labeled endocannabinoid analogues should be developed for use as metabolic trapping tracers in SPECT and PET. Another line of approach is the use of FAAH inhibitors, selectively and covalently binding to the enzyme, allowing visualization, and giving a broader spectrum of molecules for labeling with SPECT or PET isotopes. The drawback of using inhibitors is that only differences in regional enzyme density can be visualized without providing information on the functionality of the enzyme.

3. Conclusions

The aim of this study was to develop tracers for in vivo evaluation of FAAH in the brain using metabolic trapping. For this approach two iodinated AEA analogues were synthesized and evaluated in vitro and in vivo. Compounds **2** and **4** were synthesized in good yields of, respectively, 75% and 86%. In vitro evaluation of the compounds showed that both compounds were able to inhibit the metabolization of the endogenous FAAH substrate AEA and, consequently, interact with the enzyme possibly as a substrate. Incubation with the enzyme and HPLC analysis of formed fatty acid confirmed the identity of both **2** and **4** as FAAH substrates. [^{123}I]-**2** and [^{123}I]-**4** were synthesized in moderate to low yields with acceptable radiochemical purity. The biodistribution of [^{123}I]-**2** and [^{123}I]-**4** in NMRI mice demonstrated uptake of both radioligands in the brain, but no retention of radioactivity could be shown. The high stomach values suggest dehalogenation of the radioligands. Stability studies in vitro and in vivo in mouse blood and brain confirm the susceptibility of both radioligands to dehalogenation. In conclusion, we have described for the first time the synthesis and evaluation of radioligands for the in vivo evaluation of FAAH activity in the brain. The in vitro results support the potential of [^{123}I]-**2** and [^{123}I]-**4** as a tracer for visualising brain FAAH by metabolic trapping. However, stability studies lead us to suspect that although there is brain uptake, the radioligands will not be useful for in vivo visualization of FAAH because of rapid dehalogenation. Nevertheless, they could serve as an entry point to the preparation of a wide variety of radioligands based on endocannabinoids or on FAAH inhibitors for the in vivo visualization of FAAH. Work is in progress to design more stable endocannabinoid derivatives with the aim to develop a suitable radioligand for mapping brain FAAH in vivo.

4. Experimental

4.1. General procedures and materials

All chemical reagents were obtained from commercial sources (Sigma–Aldrich Fluka, Acros Organics, Belgium) and used without further purification. Solvents were purchased from Lab-Scan Analytical Sciences (Dublin, Ireland). All reactions were conducted under N_2 atmosphere with dry solvents under anhydrous conditions. The thin-layer chromatographic analyses were performed using 200 μm silica gel with fluorescent indicator (UV_{254}) coated on plastic plates (Machery-Nagel, Germany). The spots were visualized using iodine vapours. Purification of unlabeled compounds was achieved by column chromatography with silica gel (Sigma–Aldrich, 230–400 mesh), using solvent systems indicated in the text. For mixed solvent systems, ratios are given with respect to volumes. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured with a Varian 300 MHz FT-NMR spectrometer (Department of Medicinal Chemistry, Ghent University). Signals are quoted as s (singlet), d (doublet), t (triplet), q (quartet), qu (quintet) or m (multiplet). Chemical shifts were recorded in ppm (δ) from an internal tetramethylsilane standard in chloroform-d_3. Mass spectrometry was performed on a Waters Micromass ZMD mass-spectrometer with electrospray-ionization probe. Samples were dissolved in methanol. [^{123}I]-Sodium iodide (in 0.05 M NaOH) was purchased from GE Healthcare (Belgium). High performance liquid chromatography (HPLC) purification and analyses of the radiotracer were performed using a Waters 515 HPLC pump, a Waters 2487 UV detector (204 nm), and a Ludlum 220 scaler ratemeter equipped with a GM-probe. Radioactivity of samples was measured in a Packard Cobra automated γ-counter equipped with five 1″ × 1″ NaI(Tl) crystals. Radioactivity on TLC plates was quantified using a Cyclone Storage Phosphor System (Perkin Elmer, USA). For the animal studies, male NMRI (Naval Medical Research Institute) mice were used. All animal experiments were conducted according to the regulations of Belgian law and the local Ethical Committee (ECP 07/05).

4.2. Chemistry

4.2.1. *N*-(2-Hydroxyethyl)linoleoylamide (1)

To a solution of linoleic acid (500 mg, 1.67 mmol) in dry dichloromethane (CH_2Cl_2, 15 ml) was added carbonyl diimidazole (CDI, 270.54 mg, 1.67 mmol). The reaction mixture was stirred at room temperature for 1 h. Ethanolamine (3.34 mmol, 202 μl) was added dropwise, and the reaction mixture was stirred for 3 h at room temperature. The reaction mixture was diluted with dichloromethane (30 ml) and washed two times with water and with saturated brine. The organic layer was dried over $MgSO_4$, and the solvent was evaporated under reduced pressure to give the crude product. Purification by silica gel flash chromatography (8:2 ethyl acetate/hexane) gave **1** as a white solid (420 mg) in a 78% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (3H, t, J = 6.6 Hz); 1.32 (14H, m); 1.64 (2H, qu, J = 7.5 Hz); 2.05 (4H, q, J = 6.9 Hz); 2.20 (2H, t, J = 7.2 Hz); 2.76 (2H, t, J = 6.3 Hz); 3.43 (2H, q, J = 5.4 Hz); 3.72 (2H, t, J = 5.1 Hz); 5.34 (4H, m); 5.9 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.21, 22.71, 25.77, 25.86, 27.33, 27.34, 27.34, 29.27, 29.39, 29.48, 29.75, 31.66, 36.78, 42.63, 62.62, 128.04, 128.21, 130.17, 130.38, 174.75. MS (ESI) *m/z* (% rel int.): 324.2 (100.0 $[M+1]^+$); 346.1 (28.2 $[M+Na]^+$); 647 (20 $[2M-1]^+$).

4.2.2. *N*-(2-Iodoethyl)linoleoylamide (2)

To a stirred solution of triphenylphosphine (913 mg, 3.48 mmol) and imidazole (237 mg, 3.48 mmol) in dry CH_2Cl_2 (20 ml) cooled to 0 °C was added in small portions I_2 (883 mg, 3.48 mmol) over a period of 5 min. The yellow-orange suspension was warmed to room temperature and stirred for 10 min. A solution of amide **1** (930 mg, 2.90 mmol) in dry CH_2Cl_2 (10 ml) was added dropwise, and the resulting mixture was stirred at room temperature for 3 h. The reaction mixture was washed twice with aqueous $Na_2S_2O_3$ (5%). The layers were separated and the aqueous layer extracted with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$, and the solvent was evaporated under reduced pressure to give the crude product. Purification by silica gel flash chromatography (7:3 hexane/ethyl acetate) gave **2** as a yellow solid (342 mg) in a 75% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.88 (3H, t, J = 6.9 Hz); 1.30 (14H, m); 1.64 (2H, qu, J = 7.2 Hz); 2.04 (4H, q, J = 7.2 Hz); 2.20 (2H, t, J = 7.2 Hz); 2.76 (2H, t, J = 6.0 Hz); 3.25 (2H, t, J = 6.0 Hz); 3.60 (2H, q, J = 6.0 Hz); 5.35 (4H, m); 5.80 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 6.10, 14.22, 22.71, 25.77, 25.80, 27.33, 27.34, 29.26, 29.37, 29.38, 29.48, 29.74,

31.66, 36.84, 41.76, 128.04, 128.19, 130.17, 130.36, 173.26. MS (ESI) *m/z* (% rel int.): 434.3 (5.0 $[M+1]^+$); 306.3 (100 $[M-I]^+$; 324.3 (12.2 $[(M-I)+H_2O]^+$.

4.2.3. Arachidonylethanolamide (3)

Synthesis followed the same procedure as described for **1** using arachidonic acid (1 g, 3.29 mmol) as the fatty acid. Purification by silica gel flash chromatography (6:4 ethyl acetate/hexane) gave **3** as a white solid (730 mg) in a 64% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.89 (3H, t, *J* = 6.6 Hz); 1.30 (6H, m); 1.73 (2H, qu, *J* = 7.2 Hz); 2.08 (4H, q, *J* = 6.9 Hz); 2.20 (2H, t, *J* = 7.5 Hz); 2.80 (6H, m); 3.40 (2H, q, *J* = 5.4 Hz); 3.72 (2H, t, *J* = 5.1 Hz); 5.36 (8H, m); 5.95 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.20, 22.71, 25.61, 25.61, 25.77, 26.77, 27.36, 29.45, 31.65, 36.05, 42.60, 62.55, 127.65, 127.98, 128.27, 128.41, 128.76, 129.00, 129.14, 130.68, 174.37. MS (ESI) *m/z* (% rel int.): 348.5 (100 $[M+1]^+$); 695.2 (34.0 $[2M+1]^+$); 370.4 (16.4 $[M+Na]^+$); 329.6 (14.0 $[M-H_2O]^+$.

4.2.4. *N*-(2-Iodoethyl)arachidonylamide (4)

The same procedure described for the synthesis of **2** was applied, using **3** as the added amide, resulting in the crude product. Purification by silica gel flash chromatography (7:3 hexane/ethyl acetate) gave **4** as a yellow-white solid (448 mg) in a 86% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.89 (3H, t, *J* = 7.0 Hz); 1.30 (6H, m); 1.73 (2H, qu, *J* = 7.6 Hz); 2.05 (2H, q, *J* = 7.0 Hz); 2.13 (2H, q, *J* = 7.0 Hz); 2.20 (2H, t, *J* = 7.3 Hz); 2.81 (6H, m); 3.27 (2H, t, *J* = 6.5 Hz); 3.60 (2H, q, *J* = 6.1); 5.36 (8H, m); 5.77 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 6.04, 14.23, 22.72, 25.56, 25.81, 26.80, 27.38, 29.47, 31.67, 36.12, 41.78, 127.67, 128.00, 128.29, 128.41, 128.77, 129.04, 129.15, 130.68, 172.94. MS (ESI) m/z (% rel int.): 457.9 (6.4 $[M+1]^+$); 330.5 (100 $[M-I]^+$); 347.9 (25.0 $[(M-I)+H_2O]^+$).

4.2.5. 2-(Linoleoylamido)-ethyl trifluoromethanesulfonate (5)

To a stirred solution of **1** (200 mg, 0.619 mmol) and freshly distilled triethylamine (96 μl, 0.681 mmol) in dry CH_2Cl_2 (10 ml) at −20 °C was added trifluoromethanesulfonic anhydride (112 μl, 0.681 mmol) over 30 min. The resulting opaque solution was stirred at 0 °C for 1 h in the dark. The reaction mixture was diluted with CH_2Cl_2 (30 ml) and washed with water. The organic phase was isolated and dried over $MgSO_4$, and the solvent was evaporated under reduced pressure. A yellowish oil (204 mg, 72%) was obtained and used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (3H, t, *J* = 7.0 Hz); 1.30 (14H, m); 1.56 (2H, q, *J* = 7.3 Hz); 1.98 (4H, q, *J* = 6.7 Hz); 2.28 (2H, t, *J* = 7.3 Hz); 2.70 (2H, t, *J* = 5.6 Hz); 3.50 (2H, q, *J* = 5.3 Hz,); 4.16 (2H, t, *J* = 5.0 Hz); 5.29 (4H, m). MS (ESI) *m/z* (% rel int.) 306.3 (100 $[M-OTf]^+$); 324.3 (35.6 $[(M-OTf)+H_2O]^+$).

4.2.6. 2-(Arachidonylamido)-ethyl trifluoromethanesulfonate (6)

The procedure for the synthesis of **5** was applied to **3** yielding **6** as a yellowish oil (244 mg, 88%) which was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.88 (3H, t, *J* = 7.0 Hz); 1.28 (6H, m); 1.71 (2H, q, *J* = 7.03 Hz); 2.08 (4H, dq, *J* = 7.03 and 7.62 Hz); 2.36 (2H, t, *J* = 7.62 Hz); 2.81 (6H, q, *J* = 5.85 Hz); 3.54 (2H, q, *J* = 5.27 Hz); 4.22 (2H, t, *J* = 4.98 Hz); 5.37 (8H, m). MS (ESI) *m/z* (% rel int.): 330.3 (100 $[M-OTf]^+$); 348.3 (90.0 $[(M-OTf)+H_2O]^+$).

4.3. Radiochemistry

4.3.1. *N*-(2-[^{123}I]-iodoethyl)linoleoylamide ([^{123}I]-2) and *N*-(2-[^{123}I]-iodoethyl)arachidonylamide ([^{123}I]-4)

The radiosynthesis of [^{123}I]-**2** and [^{123}I]-**4** was performed starting from the triflate precursor (compound **5** and compound **6**, respectively) followed by HPLC purification. Optimal reaction conditions were obtained with a mixture of 4 mg of compound **5** or **6** in 0.5 ml acetone at 50 °C for 30 min for [^{123}I]-**2** and at 40 °C for 30 min for [^{123}I]-**4**.

The freshly synthesized triflate precursor **5** (4 mg, 0.00879 mmol) or **6** (4 mg, 0.00835 mmol) was dissolved in dry acetone (0.5 ml). The conical reaction vial containing the reaction mixture was flushed with N_2, and [^{123}I]NaI in sodium hydroxide solution (4 μl, 0.05 M) was added. The reaction proceeded for 30 min at 50 °C for [^{123}I]-**2** and at 40 °C for [^{123}I]-**4**. The acetone was evaporated under a stream of N_2, and the reaction mixture was redissolved in acetonitrile (100 μl). The solution was injected into a HPLC system (Alltech Alltima C_{18} column, 150 × 4.6 mm, particle size 5 μm + Alltech Alltima C_{18} guard 7.5 × 4.6 mm, particle size 5 μm) and eluted with a 80:20:0.1% mixture of acetonitrile:water:formic acid at a flow rate of 1 ml/min to isolate the radiolabeled compounds [^{123}I]-**2** and [^{123}I]-**4**. The desired fraction eluted at 24–26 min for [^{123}I]-**2** and at 22–24 min for [^{123}I]-**4**, was collected and concentrated. The product was redissolved in 0.9% NaCl solution containing ethanol (8%) for biologic evaluation. Yields: [^{123}I]-**2**: 21 ± 3% (*n* = 6), [^{123}I]-**4**: 12 ± 6.3% (*n* = 4).

4.3.2. Radioanalytical data

4.3.2.1. Quality control. HPLC analysis was used to measure the chemical and radiochemical purity. 500 μl of the product fraction was re-injected into the HPLC column (Alltech Alltima C_{18} column, 150 × 10 mm, particle size 10 μm + Alltech Alltima C_{18} guard 33 × 7 mm, particle size 10 μm, 80:20:0.1% acetonitrile:water:formic acid, flow rate 6 ml/min, t_R 19.9 min for [^{123}I]-**2** and 19.4 min for [^{123}I]-**4** using ultraviolet (UV) and radioactivity detectors). The quality control did not show any radioactive impurities, with the exception of some [^{123}I]I^- (t_R 1.2 min, <10%). Only the injection peak was detectable within the UV-range.

4.3.2.2. Reference control. An aliquot of [^{123}I]-**2** or [^{123}I]-**4** was co-injected with 50 μg of compound **2** or compound **4**, respectively, into the HPLC system to confirm its identity. The radiolabeled compounds and the non-radioactive references co-eluted, confirming their identity.

4.4. In vitro evaluation

4.4.1. Compounds

Radiolabeled arachidonylethanolamide ([^{3}H]-AEA, labeled in its ethanolamine moiety, specific activity of 60 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Non-radioactive arachidonylethanolamide (AEA) and methyl arachidonoyl fluorophosphonate (MAFP) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Fatty acid-free bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (Belgium).

4.4.2. FAAH assay

The method used was based on that of Omeir et al. using [^{14}C]-AEA as substrate, and has been previously described.[39,42] Briefly, recombinant rat FAAH fused to Maltose Binding Protein (rFAAH)[46] was diluted to the appropriate assay protein concentration (1.2 μg per assay) in Tris–HCl buffer (10 mM, pH 7.6) containing 1 mM EDTA. Aliquots (165 μl) were added to glass tubes containing 10 μl of test compound **2** or **4**. Blanks contained assay buffer instead of rFAAH. [^{3}H]-AEA (25 μl, final concentration 2 μM) was added to the test tubes, and the samples were incubated for 10 min at 37 °C. After the incubation the reaction was stopped by adding 400 μl chloroform/methanol (1:1, v/v), followed by vortex mixing and centrifugation (5 min, 2500 rpm) for phase separation. Aliquots (200 μl) of the methanol/buffer phase containing the water soluble reaction products (containing [^{3}H]-ethanolamine)

were measured for tritium content by liquid scintillation spectroscopy with quench correction. Experiments were performed in threefold.

4.4.3. Analysis of data

The pooled data expressed as percentage of control activity containing the same carrier concentration were analyzed using the built-in equation 'sigmoidal dose–response (variable slope)' of the GraphPad Prism computer program (GraphPad Software Inc., San Diego, CA, USA).

4.4.4. HPLC analysis of metabolization

For the assay of metabolization, 0.22 mM of test compound **2** or **4** was incubated with 150 μg of rFAAH in Tris–HCl buffer (10 mM, pH 7.6) containing 1 mM EDTA, and 0.1% BSA (final volume of 500 μl) at 37 °C for 20 min with shaking. Blanks contained assay buffer instead of rFAAH. The reaction was terminated by adding 500 μl cold acetonitrile, followed by vortex and centrifugation (2 min, 6000 rpm) to remove the proteins. Hundred microliters of supernatant was injected into the HPLC system for analysis (Alltech GraceSmart C_{18} column, 250 × 4.6 mm, particle size 5 μm, 90:10:0.1% acetonitrile/water/formic acid, flow rate 1 ml/min, t_R 5.7 min for arachidonic acid, 6.2 min for linoleic acid, 7.6 min for **2** and 7.1 min for **4** using UV detection, 204 nm). An identical incubation with anandamide (t_R 5.4 min) was carried out to validate the method.

For the FAAH inhibition assay, 150 μg of rFAAH was preincubated for 15 min with 10 nM of MAFP in Tris–HCl buffer (10 mM, pH 7.6) containing 1 mM EDTA, and 0.1% BSA (final volume of 500 μl) at 37 °C with shaking. Next, test compound **2** or **4** was added (final concentration 0.22 mM), incubated for another 20 min at 37 °C and analyzed as above.

4.5. Brain uptake of [^{123}I]-2 and [^{123}I]-4 in mouse brain

A biodistribution study of [^{123}I]-**2** and [^{123}I]-**4** was performed in NMRI mice. Adult male NMRI mice weighing 20–25 g were injected iv in the tail vein with 4–5 μCi of [^{123}I]-**2** or [^{123}I]-**4** dissolved in 200 μl of ethanol/saline (8:92). The mice were sacrificed by cervical dislocation under isoflurane anesthesia at selected time points after injection (n = 3 per time point). Blood and urine were collected, and the brain and main organs to be examined were rapidly removed and weighed. Radioactivity of the samples was measured in a γ-counter. The uptake of radioactivity in organs and tissues was expressed as a percentage of the injected dose per gram of tissue plus or minus the standard deviation (%ID/g tissue ± SD).

4.6. Tracer dehalogenation

Tracer stability was determined in vitro and in vivo in mouse plasma and brain tissue using HPLC and thin-layer chromatographic analysis with radioactivity detection.

4.6.1. In vitro plasma and brain tracer stability

Three male NMRI mice, weighing 20–25 g were sacrificed by cervical dislocation after isoflurane anesthesia. Brain and blood were taken and placed in ice. Blood samples were collected in EDTA-coated vials, centrifuged for 5 min at 3000*g* and the plasma was separated from the pellet. Brain and plasma samples were spiked with 3 μCi of [^{123}I]-**2** or [^{123}I]-**4**. To the brain tissue, 500 μl of acetonitrile was added. The sample was homogenized and an extra 1 ml of acetonitrile was added. To the spiked plasma samples, 800 μl of acetonitrile was added. The samples of brain and plasma were centrifuged (3 min, 3000*g*) and the supernatant and pellet were separated. Radioactivity of the supernatant and pellet was measured in a Packard Cobra γ-counter to quantify the extraction efficiency. The supernatant was analyzed by radio-thin-layer chromatography (TLC, silica, 4:6 hexane/ethylacetate) and HPLC. Five hundred microliters of the supernatant was injected into a HPLC system (Alltech Alltima C_{18} column, 150 × 10 mm, particle size 10 μm + Alltech Alltima C_{18} guard 33 × 7 mm, particle size 10 μm, 80:20:0.1% acetonitrile/water/formic acid, flow rate 6 ml/min). The eluate was collected in fractions of 3 ml (=0.5 min) and counted for radioactivity in a γ-counter.

4.6.2. In vivo tracer stability

Three male white mice (20–25 g) were each injected with approximately 200 μCi of [^{123}I]-**2** dissolved in 200 μl of ethanol/saline (8:92) and sacrificed 3 min postinjection. Blood and brain were taken. Blood samples (in EDTA-coated vials) were centrifuged for 3 min at 3000*g* and the plasma was separated from the pellet. To 200 μl of plasma 800 μl of acetonitrile was added and the mixture was vortexed for 10 s followed by centrifugation for 3 min at 3000*g*. To the brain tissue, 1500 μl of acetonitrile was added. It was mixed for 30 s and centrifuged for 3 min at 3000*g*. For both plasma and brain, 500 μl of each supernatant was processed on a HPLC system (Alltech Alltima C_{18} column, 150 × 10 mm, particle size 10 μm + Alltech Alltima C_{18} guard 33 × 7 mm, particle size 10 μm, 80:20:0.1% acetonitrile/water/formic acid, flow rate 6 ml/min). The eluate was collected in fractions of 3 ml (=.5 min) and counted for radioactivity in a γ-counter.

4.6.3. Radio-thin-layer chromatography

The test solutions were spotted at 1 cm from one end of silica TLC plates (200 μm silica gel coated on plastic plates, Machery-Nagel, Germany). The plates were developed in hexane/ethylacetate (4:6) and activity on the plates was quantified using a Cyclone Storage Phosphor System. Regions of interest corresponding to authentic radiotracer, metabolite and chromatographic origin were drawn manually. R_f values were 0.64 for authentic [^{123}I]-**2** and 0.66 for authentic [^{123}I]-**4**. The percentage of authentic radiotracer was calculated as the optical density of the authentic tracer region divided by the total signal in all regions.

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