Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 295–298

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

A Lipophilic Thioflavin-T Derivative for Positron Emission Tomography (PET) Imaging of Amyloid in Brain

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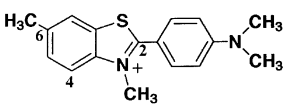
Received 24 July 2001; accepted 29 October 2001

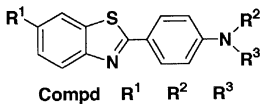
Abstract—The synthesis of a new lipophilic thioflavin-T analogue (2-[4′-(methylamino)phenyl]benzothiazole, **6**) with high affinity for amyloid is reported. Intravenous injection of [^{11}C]-labeled **6** in control mice resulted in high brain uptake. Amyloid deposits were imaged with multiphoton microscopy in the brains of living transgenic mice following the systemic injection of unlabeled **6**. [^{11}C]**6** is a promising amyloid imaging agent for Alzheimer's disease.

The development of small radiolabeled molecules for use as biological markers of β-amyloid (Aβ) deposits in Alzheimer's disease (AD) has been a goal of researchers for several years.[1] The advent of therapies that may affect Aβ deposition in AD brain[2] has added new significance to this pursuit.[3] A variety of radiolabeled Congo red, Chrysamine-G, and other analogues have been synthesized and evaluated as potential in vivo positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging probes of amyloid deposition in AD brain.[4–9] While many of these radiotracers displayed high affinity for Aβ, good brain uptake has been hindered by the presence of highly polar functional groups. We reported[10] the synthesis and initial characterization of neutral analogues of the amyloid dye thioflavin-T (ThT; Fig. 1), and a neutral radioiodinated ThT analogue also has been described.[11]

Removal of the methyl group from the heterocyclic nitrogen of ThT eliminated the positive charge from the benzothiazole ring and provided a series of lipophilic dyes (**1**–**3**) with high affinity for Aβ and good brain uptake in rodents.[10] Here, we report the results of structure-brain uptake studies that show [*N*-methyl-^{11}C]2-[4′-(methylamino)phenyl]benzothiazole ([^{11}C]**6**) has characteristics more promising than previously reported compounds.[10,11] To demonstrate that systemically administered, unlabeled, fluorescent **6** (Fig. 1) can resolve individual Aβ plaques and cerebrovascular amyloid in living transgenic mice,[12] we employed a novel high-resolution imaging technique, multiphoton microscopy. Future studies will include imaging amyloid load in transgenic mice using newly developed high-resolution microPET,[13] a technology that will provide a direct transition to PET imaging studies in human subjects.



thioflavin-T (ThT)



Compd	R^1	R^2	R^3
1	CH_3	H	H
2	CH_3	H	CH_3
3	CH_3	CH_3	CH_3
6	H	H	CH_3

Figure 1. Structural representations of the amyloid dyes thioflavin-T and neutral ThT analogues **1**–**3** and **6**.

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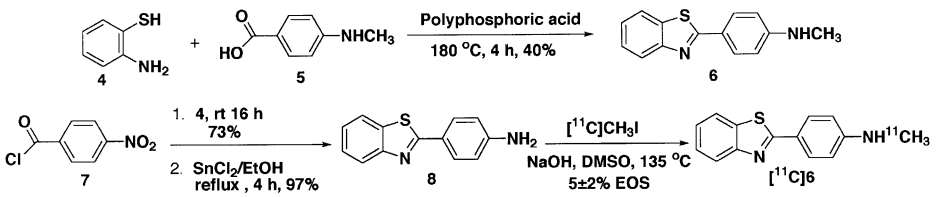

Scheme 1. Synthesis of compounds **6** and [^{11}C]**6**.

The syntheses of unlabeled[14] and carbon-11-labeled[15] **6** are shown in Scheme 1 and were achieved using literature-based methods.[10,16] Compound **6** was synthesized by direct coupling of 4-(methylamino)benzoic acid (**5**) with 2-aminothiophenol (**4**) in the presence of polyphosphoric acid at elevated temperature. The primary amine precursor for [^{11}C]-labeling (**8**) was synthesized via reduction of the intermediate 2-(4′-nitrophenyl)-benzothiazole, which was prepared from the coupling of 4-nitrobenzoyl chloride (**7**) with **4**.

The results of in vitro competition binding assays using [*N*-methyl-^{3}H]**6**[17] and synthetic aggregated Aβ(1–40) fibrils[10] are shown in Table 1 and demonstrate that the neutral analogues **2** and **6** possessed higher affinity for Aβ(1–40) than the charged parent compound, ThT. Octanol–water partition coefficients (Poct) of **2** and **6** were determined[10] at pH 7.4, and the logPoct values are given in Table 1. The logPoct value of [^{11}C]**6** is near the range of values predicted[18,19] to result in optimal brain uptake at early times following intravenous (iv) bolus injection, while the logPoct value of [^{11}C]**2** is somewhat beyond the optimum range of 1.5–2.5. Whole-brain uptake values[10] at 2 min post iv injection of [^{11}C]**2** and [^{11}C]**6** in wild-type (control) female Swiss–Webster mice are shown in Table 1, and the nearly 2-fold higher brain uptake of [^{11}C]**6** over [^{11}C]**2** supported the logPoct-based prediction. The egress of radioactivity from normal mouse brain tissue was also determined by measurement of whole brain radioactivity at 30 min following bolus iv injection (Table 1). Injection of the less lipophilic compound, [^{11}C]**6**, resulted in more rapid clearance of radioactivity from the brain and provided a 2–30 min brain concentration ratio nearly 3-fold higher than that of [^{11}C]**2**, indicative of good clearance of free and nonspecifically bound radioactivity from control mouse brain tissue.

The peripheral metabolism of [^{11}C]**6** in control mice was rapid, with unmetabolized [^{11}C]**6** representing 92% of total plasma radioactivity at 2 min after injection and 12% at 30 min.[20] All of the radiolabeled plasma metabolites of [^{11}C]**6** were polar species and were not expected to enter brain. To confirm this, mice were injected with [^{11}C]**6**, and their brains were removed at 2 and 30 min after injection and homogenized.[21] Radiolabeled species were extracted and assayed, and >95% of the radioactivity in the homogenates was found to be unmetabolized [^{11}C]**6**, indicating very low brain uptake of radiolabeled metabolites of [^{11}C]**6**.

In other experiments, three 17-month-old PS1/APP transgenic mice[12] were injected intraperitoneally (ip) with a single dose of 10 mg/kg of compound **6** in a solution of DMSO, propylene glycol, and pH 7.5 PBS (10/45/45 v/v/v). Twenty-four hours later, multiphoton fluorescence microscopy[22,23] was employed to obtain high-resolution images in the brains of these living mice using a cranial window technique.[24–26] Typical in vivo images of compound **6** in a living PS1/APP mouse are shown in Figure 2, and plaques and cerebrovascular amyloid are clearly distinguishable. After imaging with

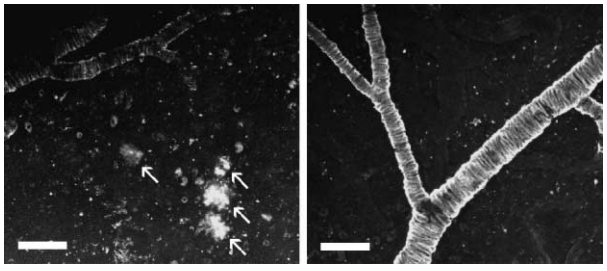

Figure 2. Multiphoton microscopic images of amyloid deposits in living PS1/APP mouse brain 24 h after injection of compound **6** (10 mg/kg). These cortical images were obtained slightly lateral to the sagittal suture and just posterior to the coronal suture through a cranial window preparation. Fluorescence was excited at 750 nm and collected at 380–480 nm. The views are projections ~300 μm deep. The image on the left reveals several bright plaques (arrows) and the faint outlines of cerebrovascular amyloid (upper left of panel). The image on the right shows a distinct amyloid-laden vessel. Note the absence of fluorescence emanating from within the vessels 24 h after the injection of compound **6**. The scale bar is 100 μm.

Table 1. Comparison of the in vitro and in vivo properties of thioflavin-T and neutral thioflavin-T analogues **2** and **6**

Compd	ThT	**2**	**6**
K_i (nM)[a] Aβ(1–40) fibrils	580 (±38)	10 (±0.5)	11 (±1)
logPoct (pH 7.4)	0.57[b]	3.4[b]	2.7
2 min mouse brain uptake (%ID/g)[c]	—	7.6 (±0.3)[b]	12.9 (±4.2)
30 min mouse brain uptake (%ID/g)[c]	—	2.8 (±0.1)[b]	1.7 (±0.3)
Ratio of 2-to-30 min mouse brain uptakes	—	2.7	7.6

[a]Values are means (±SD) of three experiments.
[b]Previously published values[10] provided for reference.
[c]Values are means (±SD) of 3–5 measurements.

6, one of the mice also received an iv injection of Texas Red-dextran (M_r 70,000, Molecular Probes), a red fluorescent dye that does not cross an intact blood–brain barrier (BBB).[22,23] Subsequent imaging showed no leakage of Texas Red-dextran across the cerebral vasculature, indicating that the BBB remained intact.

The multiphoton microscopy studies demonstrated the in vivo specificity of compound **6** for Aβ in the brains of living PS1/APP transgenic mice and provided the first reported in vivo images of amyloid deposits in the brain of transgenic mice following systemic injection of an amyloid imaging probe. This technique likely will provide a valuable experimental complement to future microPET imaging studies of [^{11}C]**6** in PS1/APP transgenic mice and assist in the interpretation of the lower resolution (2 mm FWHM) PET studies.

In summary, we report the synthesis of a new lipophilic thioflavin-T analogue, compound **6**, with high affinity for Aβ. Injection of [^{11}C]**6** in control mice resulted in high brain uptake at early time points and relatively rapid egress of radioactivity from normal brain tissue. Injection of unlabeled **6** in PS1/APP mice resulted in the visualization of both cerebral plaques and cerebrovascular amyloid deposits. Thus, [^{11}C]**6** is a promising lead radioligand for further development as a PET amyloid imaging agent.

Acknowledgements

Financial support was provided in part by grants from the Alzheimer's Association (Pioneer Award to B.T.H. and NIRG-00-2335) and the National Institute on Aging (AG08487, AG15379, and AG18402).

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14. The procedures used to prepare **6** and precursor **8** for subsequent radiolabeling were as follows: 2-Aminothiophenol (2.5 g, 20 mmol) (**4**) and 4-(methylamino)benzoic acid (**5**) (3.0 g, 20 mmol) were heated in polyphosphoric acid (10 g) at 180 °C for 4 h. After cooling, the reaction mixture was poured into 10% Na_2CO_3 solution (50 mL). The precipitated product was collected by filtration under vacuum and was purified by flash chromatography (hexane/ethyl acetate, 9:1). Subsequent de-colorization using activated carbon gave 1.9 g (40%) of **6**. ^{1}H NMR (300 MHz, acetone-d_6) δ 7.92 (d, J = 7.7 Hz, 1H), 7.88 (d, J = 7.7 Hz, 1H), 7.86 (d, J = 7.9 Hz, 2H), 7.44 (dt, J_1 = 7.7 Hz, J_2 = 2.1 Hz, 1H), 7.33 (dt, J_1 = 7.7 Hz, J_2 = 1.9 Hz, 1H), 6.70 (d, J = 7.9 Hz, 2H), 2.85 (s, 3H). HRMS m/z calcd for $C_{14}H_{12}N_2S_1(M^+)$ 240.0721, found 240.0721. For **8**: 4-Nitrobenzoyl chloride (**7**) (1.5 g, 8.0 mmol) and 2-aminothiophenol (**4**) (1.0 g, 8.0 mmol) in anhydrous benzene (20 mL) were stirred at room temperature for 16 h. Following extraction with ethyl acetate from water, the solvent was evaporated, and the residue was purified by flash chromatography (hexane: ethyl acetate, 85:15) to give 1.5 g (73%) of 2-(4′-nitrophenyl)benzothiazole as a pale-yellow solid. 2-(4′-Nitrophenyl)-benzothiazole (0.10 g, 0.40 mmol) and tin(II) chloride dihydrate (0.20 g, 0.90 mmol) in ethanol (20 mL) were then refluxed under N_2 for 4 h. Ethanol was removed, and the residue was dissolved into ethyl acetate (20 mL). The solution was washed with 1 N NaOH solution (3×20 mL) and water (3×20 mL) followed by evaporation of the solvent to give 0.10 g (97%) of **8**. ^{1}H NMR (300 MHz, acetone-d_6) δ 7.7–8.2 (m, 4H), 7.4–7.6 (m, 1H), 7.2–7.4 (m, 1H), 6.81 (d, J = 8.4 Hz, 2H).
15. Typical radiolabeling procedures were as follows: Compound **8** (1 mg) was dissolved in DMSO (0.4 mL), and 5 N NaOH (10 μL) was added. High specific activity [^{11}C]CH_3I was produced,[31] and the entire amount was bubbled through the solution. The reaction mixture was heated at 95 °C for 5 min, and the crude reaction mixture was purified by semi-preparative HPLC (Prodigy ODS-Prep) eluted with acetonitrile/aqueous buffer (55/45 v/v, 50 mM triethylammonium phosphate buffer, pH 7.2). The fraction containing [^{11}C]**6** was collected, and the product was isolated from the HPLC eluent using a C8 SepPak Plus cartridge (Waters Corp.). The cartridge was washed with 10 mL water and eluted with 1 mL ethanol to yield [^{11}C]**6** with a radiochemical purity >95% and a specific activity of 55–110 GBq/μmol at the end of synthesis (EOS). Only trace amounts of the dimethylated product were produced, as the amine precursor **8** was in ~100-fold stoichiometric excess over [$^{11/12}$C]CH_3I, and this byproduct was readily separated by the semi-preparative HPLC conditions. Analytical HPLC of [^{11}C]**6** was performed using reverse-phase chromatography [Phenomenex Prodigy ODS(3) 5 μm 250 × 4.6 mm column eluted with acetonitrile/aqueous buffer (55/45 v/v, 50 mM triethylammonium phosphate buffer, pH 7.2)]. The k' value of [^{11}C]**6** under these conditions was 5.7, and [^{11}C]**6** co-eluted with a sample of authentic **6**. In addition, normal-phase chromatography was utilized to confirm the co-elution of [^{11}C]**6** and a sample of authentic **6** [Whatman Partisil 10 250×4.6 mm 10 μm eluted with ethyl acetate/n-hexane/DEA (11.5/88/0.5 v/v/v)]. The k' value of [^{11}C]**6** and **6** under these conditions was 7.8. In-line HPLC detectors included UV detectors (Waters Corp. Model 996) and radio-HPLC detectors (Raytest Corp. Model Gabi).
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GBq of [^{3}H]CH_3I were utilized rather than [^{11}C]CH_3I. The final product ([*N*-methyl-^{3}H]**6**) was obtained in ~10% radiochemical yield with a radiochemical purity greater than 97% and a specific activity of 2.3 GBq/μmol.
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21. The procedures used to assess brain radioactivity were as follows: Female Swiss–Webster mice (20–26 g) were injected via lateral tail veins with 0.74–3.7 MBq (20–100 μCi) of high specific activity (>50 GBq/μmol) [^{11}C]**6** contained in 0.1 mL of isotonic saline solution (containing 5% ethanol). The mice were anesthetized at 2 min and 30 min post injection and killed by cardiac excision. The mouse brains were rapidly removed and divided in half down the midline of the brain. A modified Folch extraction[32] was used to prepare the brain for analysis. One half of the mouse brain (~0.2 g) was added to a 1 mL solution of $CHCl_3$/MeOH (2/1 v/v) and homogenized (Polytron, Kinematica AG) for ~30 s. The homogenized solution was filtered through a plug of glass wool. The glass wool is then washed with an additional 9 mL of $CHCl_3$/MeOH (2/1 v/v). An aqueous salt solution (2.5 mL of 0.04% $CaCl_2$, 0.034% $MgCl_2$, 0.74% KCl) was added to the filtrate, and the mixture was vortexed for 2 min followed by centrifugation for 2 min at 13,000*g*. Samples from the aqueous and organic phases (500 μL each) and the entire glass wool plug were counted using a gamma well-counter (Packard Instruments) to determine the extraction efficiency (<5% of the total radioactivity remained on the glass wool plug). An authentic standard of **6** was added to an aliquot of the organic fraction, which was analyzed by reverse-phase HPLC using methods described above.[15] It was assumed that all of the radioactivity in the aqueous fraction (<3%) was radiolabeled metabolites.
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