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Generation of clickable Pittsburgh Compound B for the detection and capture of β -amyloid in Alzheimer's Disease brain

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Abstract

The benzothiazole-aniline derivative Pittsburgh Compound B (PiB) is the prototypical amyloid affinity probe developed for the *in vivo* positron emission tomography (PET) detection of amyloid beta ($A\beta$) deposits in Alzheimer's disease (AD). Specific high-affinity binding sites for PiB have been found to vary among AD cases with comparable $A\beta$ load, and they are virtually absent on human-sequence $A\beta$ deposits in animal models, none of which develop the full phenotype of AD. PiB thus could be an informative probe for studying the pathobiology of $A\beta$, but little is known about the localization of PiB binding at the molecular or structural level. By functionalizing the 6-hydroxy position of PiB with a PEG₃ spacer and a terminal alkyne (propargyl) moiety, we have developed a clickable PiB compound that was derivatized with commercially available azide-labeled fluorophores or affinity-tags using copper-catalyzed azide-alkyne cycloaddition reactions, commonly referred to as “click” chemistry. We have determined that both the clickable PiB derivative and its fluorescently-labeled conjugate have low nanomolar binding affinities for synthetic $A\beta$ aggregates. Furthermore, the fluorescent-PiB conjugate can effectively bind $A\beta$ aggregates in human AD brain homogenates and tissue sections. By covalently coupling PiB to magnetic beads, $A\beta$ aggregates were also affinity-captured from AD brain extracts. Thus, the clickable PiB derivative described herein can be used to generate a wide variety of covalent conjugates, with applications including the fluorescence detection of $A\beta$, the ultrastructural localization of PiB binding, and the affinity capture and structural characterization of $A\beta$ and other co-factors from AD brains.

Graphical Abstract

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Supporting Information:

Supporting Information contains high resolution mass spectrometry (MS1), ¹H NMR and ¹³C NMR spectra of compound 3 (Figure S1–S3) and high-resolution mass spectrometry, ¹H NMR and ¹³C NMR spectra of compound 4 (Figure S4–6).

Keywords

Pittsburgh Compound B; PiB; 6-OH-BTA-1; benzothiazole-aniline; amyloid imaging; fluorescence; click chemistry; PEGylation; Alzheimer's disease; PET imaging; amyloid; affinity capture; histofluorescence

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder pathologically characterized by the accumulation of proteinaceous β -amyloid ($A\beta$) plaques and neurofibrillary (tau) tangles in the brain.¹ The plaques are primarily composed of the peptides $A\beta(1-40)$ and $A\beta(1-42)$, which are cleaved from the $A\beta$ -precursor protein (APP) by β -(BACE) and γ -secretases.^{1, 2} Of particular importance, the accumulation of $A\beta$ is an initiating event that precedes the development of cognitive decline by 10 or more years.³⁻⁶ For this reason, sensitive diagnostic biomarkers have been sought to detect the disease process at an early stage.

Use of radiolabeled amyloid-binding ligands in conjunction with positron emission tomography (PET) has enabled real-time *in vivo* imaging and quantification of $A\beta$ deposition for the diagnosis of AD. As this diagnostic methodology is based on $A\beta$ deposition rather than cognitive deficits, it can be used to diagnose the early-stage or pre-symptomatic forms of AD.⁷⁻¹⁰ Such amyloid-binding radioligands can also be used to monitor disease progression in real-time and quantify the effects of novel drug therapies or treatment regimens.¹¹

One of the earliest and most studied amyloid tracers is Pittsburgh Compound B (PiB),¹²⁻¹⁵ also referred to by its chemical abbreviation, 6-OH-BTA-1.¹⁵ Originally conceived in the search for high-affinity amyloid ligands, PiB was developed from the amyloid-binding dye Thioflavin T. PiB exhibits a high binding affinity (<5 nM)¹⁵ for both synthetic and brain-derived $A\beta$ aggregates while displaying excellent selectivity for $A\beta$ over tau neurofibrillary tangles^{11, 16} and other pathologic misfolded protein aggregates. A critical feature of PiB is that it exhibits a range of binding affinities towards different species of $A\beta$ aggregates depending on the surrounding chemical environment. In addition, aggregated $A\beta$ from the AD brain has significantly more high-affinity PiB binding sites than does $A\beta$ that is aggregated *in vitro*.^{13, 17} Surprisingly, even though abundant, human-sequence $A\beta$ accumulates in the brains of amyloid precursor protein (APP)-transgenic mouse models and

senescent nonhuman primates, the A β lesions in these animals are almost entirely devoid of high-affinity binding sites for PiB.^{13, 18, 19} This discovery is remarkable in that no nonhuman species has yet been found to manifest an equivalent form of Alzheimer's dementia.^{18, 20, 21} Taken together, these findings strongly suggest that high-affinity PiB binding can serve as an informative indicator of the conformational structure and pathogenicity of A β .^{18, 19, 22, 23} However, major barriers to addressing this question have been the difficulty in visualizing PiB binding at the cellular and molecular levels, and in selectively enriching A β with high PiB affinity directly from human AD brain extracts.

To overcome these limitations, we have developed and characterized a clickable derivative of PiB for the detection and capture of pathogenic "PiB-positive" A β species in human AD brain tissue. The concept of click chemistry²⁴ envisions a robust synthetic transformation that can be performed in a wide variety of aqueous and organic solvents, without the need for specialized conditions such as an inert atmosphere or elevated temperatures. The copper-catalyzed azide/alkyne cycloaddition (CuAAC) is a popular click reaction due in large part to the commercial availability of numerous alkyne and azide-labeled reagents (e.g., fluorophores, proteins, enrichment media, quantum dots, gold nanoparticles, and more). Therefore, our clickable PiB probe creates an ideal synthetic platform from which structurally and functionally diverse covalent conjugates can be prepared by a facile, single-step reaction. Notably, the phenol moiety on PiB was ideal for the attachment of the propargyl-PEG₃ group in near-quantitative yields using a cesium carbonate- (Cs₂CO₃) promoted alkylation with alkyl halides.^{25, 26} We also chose to incorporate a polyethylene glycol (PEG) linker between the PiB pharmacophore and the terminal alkyne (propargyl) click handle to better accommodate the addition of bulky groups without attenuating binding affinity. This clickable PiB probe sets the foundation for the characterization of diverse amyloid conformations in AD, and may prove invaluable for determining the underlying relationship between A β conformation and pathogenicity.

Results and Discussion

Synthesis of a clickable PIB derivative

PiB (Figure 1, compound **2**) is the prototypical A β -binding agent developed for *in vivo* PET amyloid imaging and diagnosis of AD. Initially synthesized²⁷ in the search for high-affinity and brain-penetrant amyloid probes, PiB (**2**) was based on a charge-neutral derivative of Thioflavin T (ThT) (Figure 1, compound **1**).²⁷ It was found that removal of the benzothiazolium methyl group on the ThT scaffold afforded a series of charge-neutral benzothiazole-aniline (BTA) ligands with vastly increased binding affinities for aggregated A β (1–40).^{12, 15, 27, 28} In the case of PiB, the binding affinity increased ca. 200-fold from that of ThT (K_d =890 nM),²⁷ with a reported K_d of 4.3 nM.¹⁵ Unlike the cationic ThT,¹⁵ these new BTA derivatives such as PiB were found to easily penetrate the blood brain barrier, a necessary requirement for a putative imaging ligand for cerebral amyloid.²⁸

Subsequent structure-activity relationship studies revealed that the introduction of a hydroxyl group at the 6-position of the benzothiazole ring (Figure 1, compound **2**) resulted in brain penetration and clearance kinetics that were ideal for *in vivo* PET imaging,¹⁵ without affecting the high-affinity binding to A β aggregates.^{15, 27} More recent evidence has

shown that functionalization of the 6-hydroxy position with polyethylene glycol (PEG) chains of various lengths does not perturb the high-affinity binding.^{29, 30} As a result, our synthetic strategy towards the development of a clickable PiB derivative envisaged the addition of a PEG₃ linker at the 6-hydroxy position of the benzothiazole ring (Figure 1). The presence of a phenolic moiety ensured that such a modification could be easily accomplished in near-quantitative yield using a cesium carbonate- (Cs₂CO₃) promoted alkylation with alkyl halides.^{25, 26}

Our clickable PiB derivative was prepared by O-alkylation of the 6-hydroxy position of PiB with the commercially available reagent propargyl-PEG₃-bromide. This simple synthetic approach yielded the clickable PiB derivative 6-(propargyl-PEG₃)-BTA-1 (compound **3**, Figure 1). The use of this so-called “click” reaction allows for the reliable one-step covalent conjugation of **3** with commercially available azide-labeled reporter- or affinity-groups, including fluorophores and magnetic beads. To this end, preparative CuAAC reactions were used to generate the fluorescently-labeled PiB conjugate **4** and the covalent magnetic bead PiB conjugate **5**, as outlined in Figure 2.

***In Vitro* Compound Binding Assays with aggregated A β**

To be useful as amyloid-binding probes, clickable PiB (**3**) and the fluorescently-labeled conjugate (**4**) must bind aggregated A β with high affinity. The first test of these compounds was to ensure that the aforementioned chemical modifications did not disrupt the high-affinity binding of **3** or **4** to A β aggregates. The fluorescent nature of these compounds permitted the use of centrifugation-based fluorescence binding assays^{17, 31} to determine their relative binding constants to synthetic A β (1–40) fibrils, while avoiding the significant hazards and costs associated with traditional radioligand binding assay techniques. Unlike other intrinsically fluorescent amyloid ligands, PiB (**2**) does not exhibit significant changes in fluorescence intensity or emission spectra when bound to aggregated A β .^{31, 32} This allowed for the straightforward interpretation of fluorescence binding assay data, as fluorescence intensity was a direct measurement of the amount of bound compound.

The binding affinities and fluorescence maxima of the compounds (**2–4**) investigated herein are summarized in Table 1. A saturation binding isotherm of the clicked fluorescently-labeled PiB (**4**) (Figure 3A) with A β (1–40) aggregates was used to derive a binding affinity (K_d) of 164.7 nM using a one-site specific binding model. It should be noted that while these fluorescence binding assay techniques accurately convey the relative binding affinities of these compounds, the absolute binding affinities do not precisely match those obtained using traditional radioligand binding assay techniques.^{31, 32} For example, when the PiB derivative BTA-1 was analyzed with a traditional radioligand binding assay, the reported binding affinity (K_i) was 19.5 nM.³¹ However, when analyzed with a fluorescence-based saturation binding assay, the same authors reported a significantly lower binding affinity of 5230 nM.³¹ Although the precise reasons for this disparity are currently unclear, it is assumed that fluorescence-based detection is simply less sensitive than that of radioactivity, and may not reliably detect the highest-affinity binding interactions.^{17, 31, 32} As a result, the somewhat lower binding affinities of PiB and its novel derivatives reported herein are likely a result of the fluorescence-based detection methods that we employed.

To determine the binding affinities (K_i) of PiB (**2**) and its clickable derivative **3**, we chose a fluorescence competition binding assay (Figure 3B) using the fluorescently-labeled PiB derivative **4** as the reporter ligand in the hopes that the fluorophore-labeled reporter would increase assay sensitivity. As summarized in Table 1, the fluorescence spectra of the reporter (**4**) and competitor ligands (**2** and **3**) did not appreciably overlap, allowing the compounds to be used simultaneously in the same assay. Utilizing centrifugation binding assay techniques,¹⁷ the competition binding assays demonstrated the ability of both **2** and **3** to completely displace the binding of the fluorescently-labeled reporter ligand **4** to aggregated A β (1–40) (Figure 3B). These data likewise demonstrated the specificity of **4** for synthetic A β (1–40) aggregates, and were used to derive binding affinities for **2** (K_i =678.4 nM) and **3** (K_i =264.7 nM) (Table 1).

When the affinities of **2** and its clickable derivative **3** (Table 1) are compared, it is clear that the propargyl-PEG3- group of **3** does not perturb the high-affinity binding to synthetic A β fibrils. In fact, these data suggest that clickable PiB has a slightly higher binding affinity than PiB itself, although these results could be explained in terms of increased aqueous solubility imparted by the hydrophilic PEG linker. Given that fluorescence-based binding assays preserve the relative rank-order of compound binding affinities,^{31, 32} these data indicate that clickable PiB retained the high binding affinity of its precursor, PiB.

Overall, these data demonstrate that **3** and the fluorescently-labeled **4** retained their ability to bind A β (1–40) aggregates with high affinity and specificity. Additionally, these binding studies support our hypothesis that the PEG₃ linker of **3** was successful in minimizing the steric effects of the bulky 5/6-carboxyrhodamine 110 fluorescent tag on compound **4**, consistent with published reports demonstrating the tolerance of its high-affinity binding characteristics to the addition of sterically bulky groups and PEG linkers.^{29, 30, 33} Thus, the clickable PiB derivative **3** exhibits potent binding to aggregated A β , and is an ideal platform from which to develop novel high-affinity covalent conjugates of PiB.

Staining of AD brain sections with fluorescent clicked PiB and the A β antibody 6E10

To determine the ability of the fluorescently-labeled clicked PiB (**4**) to bind AD brain-derived A β plaques, formalin-fixed tissue sections from the frontal cortex of human AD cases were stained with 100 nM of **4** and examined by fluorescence microscopy. Fluorescently-labeled PiB strongly stained amyloid plaques in AD brain tissue (Figure 4A and 4B) in a pattern reminiscent of PiB itself^{15, 34} and its fluorescent derivative 6-CN-PiB.^{13, 35, 36} After staining with the fluorescent PiB conjugate **4**, the same tissue sections were then de-coverslipped and immunostained with the A β antibody 6E10. The sequential fluorescent clicked PiB labeling and immunohistochemistry demonstrated that compound **4** selectively binds to A β plaques (Figure 4A and 4B). When the same section is imaged in the red channel (middle panel, Figure 4A), the clicked PiB **4** fluorescence signal disappears, and only the background autofluorescence of lipofuscin (which is prominent in the aged human brain³⁷) remains. At the 100 nanomolar concentration, the fluorescent PiB derivative **4** does not appear to stain all A β plaques in the human AD brain; rather, it strongly binds to dense A β plaque cores, but only weakly or not at all to diffuse deposits, consistent with the hypothesis that PiB is selective for specific structural variants of A β .²²

Fluorescence binding assays with AD brain extracts

To further assess whether the fluorescently labeled PiB conjugate selectively binds A β in the AD brain, we performed a comparative binding assay using postmortem brain extracts from control and AD cases. Compound **4** was used in a single-concentration (100 nanomolar) centrifugation-based fluorescence binding assay^{17, 31} to compare the relative binding to control and AD brain extracts (Figure 4C). Due in part to the strong autofluorescence of whole brain homogenates at the wavelengths used to analyze fluorescent clicked PiB (Ex: 485 nm, Em: 528 nm), we decided to use an SDS fractionation technique³⁸ to enrich for PiB binding sites and remove unnecessary insoluble cellular debris.

For this experiment, 100 μ g total protein of control ($n=7$) and AD ($n=7$) SDS brain extracts was incubated with a fixed concentration (100 nM) of **4**, and the bound fraction was isolated by ultracentrifugation at $150,000 \times g$. The resuspended pellets were then analyzed by fluorescence spectroscopy (Ex: 485 nm, Em: 528 nm) to determine the relative amounts of compound bound to the tissue extracts (Figure 4C). Despite the high background autofluorescence of brain tissue homogenates in the 485 nm excitation and 528 nm emission range, we were still able to discern a statistically significant ($p = 4.9 \times 10^{-5}$) increase in the binding of **4** to AD brain extracts relative to control brain extracts (Figure 4C). On average, the AD cases bound 4.46-fold more fluorescent clicked PiB than the control cases, demonstrating that **4** binds AD brain-derived A β aggregates in addition to those prepared from synthetic A β (1–40) peptide *in vitro* (Figure 3).

Affinity Capture of A β aggregates from AD brain with a magnetic bead-PiB conjugate

To investigate the possibility of using PiB as an affinity-capture ligand for A β aggregates from human brain, clickable PiB was conjugated to 1 μ m diameter azide-coated magnetic beads to generate the magnetic bead-PiB conjugate **5** (Figure 2). To conclusively demonstrate complete derivatization of the magnetic beads, the reaction supernatant was analyzed by fluorescence spectroscopy to show the disappearance of free clickable PiB compound from solution (Figure 5A). In contrast to the sham labeling reaction, the fluorescence signal of the bead conjugation reaction declined to ca. 10% of its initial intensity over the course of several days (Figure 5A), indicating successful and near-complete labeling of the magnetic beads with **3**. As these same beads were subjected to two additional conjugation reactions using a 5-fold molar excess of **3**, it can be assumed that the extent of bead labeling approached 100%.

To characterize the ability of **5** to bind A β aggregates from human brain, affinity pulldown assays were performed as previously described^{39–41} and the bead eluates were analyzed by dot blot. Relative to bead controls, densitometry analysis revealed a significant enrichment ($p = 1.82 \times 10^{-6}$) of A β in the PiB-bead pulldowns from AD brain extracts (Figure 5C). These data demonstrate the ability of the PiB-magnetic bead conjugate **5** to bind and enrich A β aggregates directly from AD brain extracts.

Conclusion

As a proof-of-principle, we demonstrated that clickable PiB and its fluorescently-labeled conjugate **4** retained the ability to bind both synthetic and brain-derived A β aggregates with high affinity and specificity. As summarized in Table 1, the nanomolar affinities of **3** and **4** with synthetic A β (1–40) aggregates demonstrated that the propargyl-PEG₃ linker at the 6-hydroxy position of the PiB scaffold not only preserved its high-affinity binding, but also served to minimize the steric interference of bulky groups added via the CuAAC click reaction, including the 5/6-carboxyrhodamine 110 fluorophore of compound **4** and the 1 μ m-diameter magnetic beads of conjugate **5**. Likewise, the ability of **4** to stain immunohistochemically confirmed A β plaques in AD brain sections provided further evidence of its ability to bind brain-derived A β aggregates. Moreover, the biochemical binding assays with AD brain extracts further supported the ability of **4** to bind A β aggregates from AD brain.

The ability of the magnetic bead-PiB conjugate **5** to capture and enrich A β aggregates from AD brain is perhaps the most promising evidence in support of these novel compounds. PiB staining of AD brain sections suggests that these PiB derivatives are able to discriminate between different conformational strains or structural variants of A β ^{13, 14, 19, 22, 31} based on the relative abundance of high-affinity PiB binding sites in specific A β plaques.^{12, 19, 22} Future studies using novel clicked PiB derivatives to interrogate the underlying structural determinants of A β pathogenicity and neurotoxicity could help to unlock the molecular mechanisms of neurodegenerative diseases like AD. For example, when conjugated to enrichment media such as magnetic beads, clickable PiB may enable the selective isolation of disease-relevant A β species that express only high-affinity PiB binding sites,^{13, 19} and which are hypothesized to play a central role in the pathophysiology and etiology of AD. Moreover, the development of clicked PiB-nanogold conjugates may allow for high-resolution electron microscopic imaging and ultrastructural analysis of disease-specific A β aggregates.^{19, 22} These compounds could also prove useful for the enrichment and subsequent analysis of AD-relevant structural variants of A β , as well as the identification of previously unknown co-aggregating proteins from AD brain by mass spectrometry analysis.

Ultimately, the presence of an alkyne “click” handle on our clickable PiB derivative allows for the facile generation of a wide range of covalent PiB conjugates. For enhanced fluorescence detection and imaging of pathologic A β aggregates in AD brain tissue, for example, conjugation to near-infrared (NIR) fluorophores or quantum dots could prove ideal,³⁰ as there is little to no tissue autofluorescence in the far-red or NIR regions of the electromagnetic spectrum.^{42, 43}

Lastly, the advent of non-radioactive A β imaging technologies may allow for the use of non-invasive fluorescence-based detection of A β in the retina or lens of the eye.^{44–47} Ocular A β imaging could obviate the need for expensive and short-lived radiolabeled probes and PET imaging equipment, instead relying on relatively inexpensive retinal or lens fluorescence imagers.^{44–48} Further reducing the complexity of the diagnostic procedure, ocular amyloid detection probes can be orally ingested or topically applied.^{46, 47} In contrast to promiscuous curcumin-based fluorescent probes,⁴⁹ novel fluorescent clicked PiB derivatives have the

potential for significantly higher affinity and selectivity towards A β *in vivo*. Therefore, fluorescent clicked PiB derivatives could also provide a viable alternative to more hazardous radioactivity-based PiB PET imaging studies.

Given its synthetic versatility and high binding affinity, our clickable PiB derivative is an ideal synthetic platform for the generation of a wide range of novel PiB probes. We propose that clickable PiB provides a novel and useful means of generating high-affinity covalent PiB conjugates with applications ranging from fluorescence imaging and detection to ultrastructural examination and the affinity capture and conformational analysis of A β or co-aggregating proteins in the AD brain.

Experimental Procedures

Materials

All reagents for compound synthesis were used as supplied, and all solvents were ACS grade or better. Anhydrous solvents were stored over activated 4Å molecular sieves (Sigma Aldrich, 208604–1KG). The starting material 6-OH-BTA-1 (PiB) was obtained from ABX Biochemicals (Radeberg, Germany). The reagents propargyl-PEG₃-bromide (BP-22738) and 5/6-carboxyrhodamine 110-PEG₃-azide (BP-22478) were obtained from BroadPharm Inc. (San Diego, California).

Silica gel (pore size 60 Å, 200–400 mesh particle size) for column chromatography was obtained from Sigma Aldrich (Sigma Aldrich, 288549–500G). Analytical thin-layer chromatography was performed using glass-backed silica gel 60 F₂₅₄ TLC plates (Millipore, 1057150001) and aluminum-backed C18-W (RP-18W) silica F₂₅₄ TLC plates (Sorbtech, 2733167). Preparative thin-layer chromatography (pTLC) was performed using glass-backed C18-W (RP-18W) silica F₂₅₄ pTLC plates (Sorbtech, 2717124). Before use, pTLC plates were pre-washed with an overnight immersion in methanol followed by developing twice in methanol. The pTLC plates were then air-dried and activated at 120 °C for 2 hours, after which they were stored in a desiccator until use. All 96-well plate-based fluorescence intensity measurements were recorded using a Biotek Synergy 4 plate reader (BioTek Instruments) using the xenon flash bulb.

Synthesis of the clickable PiB derivative 6-(propargyl-PEG₃)-BTA-1 (3)

Cesium carbonate (Cs₂CO₃) (318 mg, 0.976 mmol, 2.5 eq) was added to a stirred solution of 6-OH-BTA-1 (2) (100 mg, 0.390 mmol) in 50 ml of anhydrous acetonitrile (CH₃CN) under argon. The reaction mixture was stirred at room temperature for 10 min, after which a solution of propargyl-PEG₃-bromide (117.6 mg, 0.468 mmol, 1.2 eq) was added as a solution in 1 ml of anhydrous CH₃CN. The reaction mix was heated to reflux at ~80 °C and stirred for 45 min or until complete by TLC analysis (7:3 ethyl acetate/hexanes). The reaction mixture was cooled to room temperature and filtered to remove inorganic solids. The filter cake was washed several times with dichloromethane (DCM), after which the filtrate was concentrated *in vacuo*. The residue was brought up in DCM (100 ml) and washed with water (4 × 20 ml), brine (2 × 20 ml), and then dried over MgSO₄ and concentrated *in vacuo* to afford crude 6-(propargyl-PEG₃)-BTA-1 (3).

Crude **3** was quadruple-purified using normal phase silica gel column chromatography (1:1–7:3 ethyl acetate/hexanes), and vacuum-desiccated to yield 158.1 mg of 6-(propargyl-PEG₃)-BTA-1 (**3**) (95% yield). Compound purity and identity were confirmed with high-resolution mass spectrometry (MS1) analysis (diluted in 1:1 methanol/acetone + 0.1% formic acid) and ¹H- and ¹³C-NMR analysis in deuterated chloroform (CDCl₃) (Sigma Aldrich, 151858-10×0.5ML). Based on the TLC, MS1 and NMR analyses, compound **3** was determined to be ~95% pure, with few if any contaminant peaks observed in the NMR and MS1 spectra. Monoisotopic Mass: 426.1613. Calculated [M+H]⁺: 427.1692; observed [M+H]⁺: 427.1721. The mass spectrometry analysis was performed in positive-ion mode on an LTQ-Orbitrap hybrid mass spectrometer with the MS1 spectra collected at a resolution of 100,000 f.w.h.m. High resolution MS1, ¹H and ¹³C NMR spectra of compound **3** in supporting information (Figures S1–S3).

Synthesis of 5/6-carboxyrhodamine 110-labeled PiB (**4**) by preparative CuAAC reaction

Preparative copper-catalyzed azide-alkyne cycloaddition (CuAAC) “click” chemistry was used to prepare a covalent 5/6-carboxyrhodamine 110 conjugate of the clickable PiB compound 6-(propargyl-PEG₃)-BTA-1 (**3**). To a solution of 6-(propargyl-PEG₃)-BTA-1 (**3**) (12.37 mg, 0.02901 mmol) in 1 ml tetrahydrofuran (THF), 5 ml water, 0.75 ml tert-butanol, 5 ml methanol (MeOH) and 2 ml dimethyl sulfoxide (DMSO) was added 5/6-carboxyrhodamine 110-PEG₃-azide (25 mg, 0.04351 mmol, 1.5 eq). A pre-mixed aqueous solution (167 µl) of 20 mM CuSO₄ (0.83 mg, 0.115 eq) and 100 mM tris(3-hydroxypropyl)triazolylmethylamine (THPTA) (8.85 mg, 0.575 eq) was added to the reaction, followed by 103 µl of a freshly prepared aqueous solution of 100 mM sodium ascorbate (2.07 mg, 0.36 eq). The vial was purged with argon, sealed, and stirred for 48 hours at room temperature. The reaction mixture was concentrated *in vacuo* and brought up in 1 ml of DMSO and diluted with 15 ml of 0.1% trifluoroacetic acid in water (pH ~3) and desalted over a 1 gram tC18 Sep-Pak SPE cartridge (Waters, WAT036795). The tC18 SPE cartridge was washed with water (2 × 6 ml) and the product was eluted with MeOH (2 × 6 ml) and neutralized with 0.1% (w/v) NH₄OH in water to pH ~7. The eluate was concentrated *in vacuo* to afford the crude fluorescently-labeled 5/6-carboxyrhodamine 110-BTA-1 conjugate **4**. The clicked fluorescent PiB conjugate **4** was further purified using a combination of 1 gram tC18 Sep-Pak SPE cartridges and RP-18W preparative TLC (pTLC) plates. Following synthesis of **4**, the crude reaction residue was desalted using a 1 gram tC18 Sep-Pak SPE cartridge and subsequently thrice-purified by RP-18W pTLC developed in 7:3 methanol/H₂O + 100 mM ammonium acetate pH 5. The pTLC product bands were extracted with methanol, filtered through a plug of celite and concentrated *in vacuo* to afford purified **4**. Compound **4** was purified a final time over a 1 gram tC18 Sep-Pak SPE cartridge, eluting in LC-MS grade methanol (Fisher Chemical, A456-4). The purified product was then vacuum-desiccated to remove trace solvents, affording 24.68 mg (85% yield) of **4**. Compound purity and identity were confirmed with high-resolution MS1 (Figure S4) analysis (diluted in methanol + 0.1% formic acid) and ¹H- and ¹³C-NMR analyses in deuterated dimethyl sulfoxide (DMSO-d₆) (Sigma Aldrich, 156914-1G). It should be noted that in protic solvents (e.g. water, alcohols, ethylene glycol, acetic acid, etc.), rhodamine exists in the zwitterionic and cationic forms, which exhibit strong fluorescence. However, in aprotic solvents such as DMSO, rhodamine exists in a non-fluorescent neutral lactone

form⁵⁰ and thus in DMSO it is likely that the rhodamine fluorophore in compound **4** exists in zwitterionic, cationic and lactone forms simultaneously. This would convolute the ¹H and ¹³C NMR spectra by overlaying similar, but slightly different peaks, causing asymmetry in the spectra (Figures S5–S6). Based on RP-18W TLC, MS1 and NMR analysis, compound **4** is ~95% pure, with few contaminant peaks observed in the MS1 spectra. Monoisotopic Mass: 1000.3789 Da. Calculated [M+2H]²⁺: 501.1975 observed [M+2H]²⁺: 501.2198. The mass spectrometry analysis was performed in positive-ion mode on an LTQ-Orbitrap hybrid mass spectrometer with the MS1 spectra collected at a resolution of 100,000 f.w.h.m (Figure S4).

Synthesis of the magnetic bead-PiB conjugate (**5**) by preparative CuAAC reaction

6-(propargyl-PEG₃)-BTA-1 (**3**) was covalently coupled to 1 µm-diameter azido-PEG₃-functionalized superparamagnetic beads (Click Chemistry Tools, #1036-1) made of a highly crosslinked proprietary (non-styrene) polymer matrix using several consecutive CuAAC click reactions. Briefly, 5 mg (150–250 nmol azide equivalent) of azido-PEG₃-magnetic beads were transferred to a 1.5 ml screw-cap polypropylene tube and resuspended in 425 µl of 10% methanol (MeOH) in water. To this suspension was added 10.5 µl of 24 mM (252 nmol, 1.26 eq) 6-(propargyl-PEG₃)-BTA-1 (**3**) in DMSO, 30 µl of a pre-mixed aqueous solution of 20 mM CuSO₄ and 100 mM THPTA, followed by 35 µl of a freshly prepared aqueous solution of 100 mM sodium ascorbate. The tube was purged with argon, tightly capped, and incubated for 5 days at room temperature with rotation. For comparison purposes, a sham bead conjugation reaction was prepared with everything except the copper sulfate (CuSO₄) catalyst, to prevent the conjugation reaction from proceeding. To monitor reaction progress, 20 µl aliquots of the reaction supernatants were taken at different time-points for subsequent analysis by fluorescence spectroscopy.

Two subsequent labeling reactions were performed on the same 5 mg aliquot of beads using a 5-fold excess of compound **3** to ensure complete labeling of the magnetic beads. After completion of the bead conjugation reactions, the bead supernatants were decanted and the PiB-labeled magnetic beads (**5**) were extensively washed with 10% methanol in water (5 × 1 ml), water (4 × 1 ml), and 1× PBS pH 7.4 (3 × 1 ml). The beads were resuspended in 1× PBS with 0.05% (w/v) sodium azide to a bead concentration of 10 mg/ml, and stored at 4°C.

Fluorescence analysis of the bead conjugation reactions

To monitor the progress of the azide-magnetic bead conjugation reaction, the concentration of free (unconjugated) 6-(propargyl-PEG₃)-BTA-1 in the bead-free reaction supernatant was analyzed by fluorescence spectroscopy at wavelengths corresponding to the intrinsic fluorescence spectra of the clickable PiB compound (**3**), as summarized in Table 1.

Briefly, 20 µl aliquots of the reaction supernatants were taken from both the real and sham (without CuSO₄ catalyst) conjugation reactions at different time points. A magnetic stand was used to separate the magnetic beads from the reaction supernatant, ensuring that only unconjugated clickable PiB (**3**) would be measured. To prevent the reaction from continuing, the aliquots were quenched with the addition of EDTA to a final concentration of 15 mM. 10

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μ l of the quenched reaction aliquots were diluted with 190 μ l of 1 $\times$ PBS and transferred to a black flat-bottom polypropylene 96-well plate (sc-204462, Santa Cruz Biotechnology). Fluorescence intensity was measured on a Biotek Synergy 4 plate reader using monochromators at 348 nm (5 nm bandwidth) excitation and 430 nm (5 nm bandwidth) emission, which corresponds to the intrinsic fluorescence maxima of the clickable PiB compound **3**.

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The extent of the bead labeling was determined by the relative decrease in the fluorescence intensity signal corresponding to the disappearance of the unconjugated clickable PiB compound in the bead-free reaction supernatants. As a control, the sham bead conjugation reaction without the CuSO₄ catalyst was also analyzed and plotted on the same graph. As shown in Figure 5, the fluorescence intensity of reaction supernatant from the sham reaction remained unchanged while that of the bead labeling reaction steadily decreased over time.

Preparation of synthetic A β aggregates

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A β (1–40) aggregates were prepared from synthetic A β (1–40) peptide (Anaspec, AS-24236) using a protocol adapted from Evans et al.⁵¹. Briefly, 1 mg of lyophilized A β (1–40) peptide was dissolved in 80 μ l of 1% (w/v) NH₄OH solution with the aid of gentle vortexing over 2 minutes. The peptide solution was diluted to a concentration of 1 mg/ml with 0.2 μ m-filtered 1 $\times$ PBS (pH 7.4), and neutralized to pH 7–8 with 10% HCl. Sodium azide was added to a final concentration of 0.05% (w/v) to prevent bacterial growth. To the peptide solution was added a magnetic “flea” stir bar, and the solution was stirred at 500 RPM for 7 days at 37 ^oC to induce fibril formation. The vial was vortexed and sonicated daily to remove the gelatinous residue that formed at the air-water interface. The synthetic A β (1–40) fibril solution was divided into 50 μ l aliquots in 0.5 ml polypropylene tubes and stored at –80 ^oC until use.

Preparation of brain homogenates and extracts

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For the affinity-capture experiments and compound binding assays in brain samples, postmortem human brain homogenates were prepared and detergent-fractionated to enrich the PiB-binding fraction as previously described.³⁸ Briefly, post-mortem frontal cortical tissue was Dounce-homogenized in 4 ml/g (25% w/v) of ice-cold Tris-buffered saline (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) with 1 $\times$ HALT (Pierce) protease inhibitor cocktail. Following homogenization, sodium dodecyl sulfate (SDS) was added to a final concentration of 2% (w/v), and the homogenates were sonicated (Sonic Dismembrator System, Fisher Scientific) with three 5-s pulses at 30% amplitude using a microtip probe. Protein concentrations were determined using the bicinchoninic acid (BCA) method (Pierce). The homogenates were centrifuged at 15,000 $\times$ g for 10 minutes at 10^oC and the cloudy supernatants and soft cream-colored pellets were combined as described³⁸ and transferred to fresh tubes. This fraction was defined as the SDS-extract. The remaining compact dark pellet was discarded, as it was previously determined to contain little PiB-binding activity.³⁸

Affinity capture of A β aggregates from human brain extracts

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A β aggregates were affinity-captured from the SDS-extracts of postmortem human brain tissue with magnetic beads covalently modified with clickable PiB. Immediately before each

affinity capture experiment, the control (azido-PEG3-labeled) and PiB-labeled magnetic beads (**5**) were dispersed with 5 minutes of high-intensity water bath sonication (Elmasonic E70H, Elma). Sonication dispersed any aggregated or clumped beads and ensured a monodisperse bead suspension, which was critical for reducing nonspecific binding in the control beads and for ensuring a high yield of captured A β in the PiB beads.

Briefly, 100 μ g of the SDS-extracts were diluted to 200 μ l with capture buffer (1 $\times$ PBS pH 7.4, 1% Triton X-100, 0.1% Tween 20, and 0.5% (w/v) BSA). For each affinity-capture experiment, 0.25 mg of PiB-labeled magnetic beads and unreacted control beads in 50 μ l of capture buffer were combined with 100 μ g of the SDS-extracts diluted to 200 μ l with capture buffer and incubated for 2 hours at room temperature with rotation. Using a magnetic stand, the supernatants were decanted and the beads were washed with 3 $\times$ 200 μ l of PBS with 0.1% Tween 20 (PBST) and transferred to new 0.5 ml tubes. The beads were eluted in 50 μ l of 2% SDS in TBS at 95 $^{\circ}$ C for 20 minutes. Bead eluates were transferred to new 0.5 ml tubes, diluted with 150 μ l of TBS and immediately subjected to dot blot analysis.

Dot blot analysis of affinity-capture experiments

Bead eluates were applied to pre-wetted, 0.2 μ m nitrocellulose membranes with the assistance of light vacuum suction through a fritted glass filter, and blocked with StartingBlockTM (TBS) blocking buffer (ThermoFisher Scientific, 37542) + 0.05% Tween 20 for 30 min at room temperature. Membranes were probed overnight with a 1:500 dilution of antibody 6E10 (in-house mouse mAb) and 4G8 (Covance, SIG-39220) overnight at 4 $^{\circ}$ C, followed by 1:20,000 Goat Anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor[®] 680 conjugate (ThermoFisher Scientific, A-21058) for 1 hour at room temperature. Membranes were washed 3 $\times$ 10 min in TBST (TBS + 0.1% Tween 20), 2 $\times$ 5 min in TBS, and imaged with the infrared Odyssey Imaging System (LI-COR Biosciences). Densitometry analysis was performed using the Odyssey Imaging software (LI-COR Biosciences).

In vitro assays of compound binding to synthetic A β (1–40) aggregates

Fluorescence intensity and emission spectra were experimentally determined in 96-well plate format before binding assays were performed. Fluorescence binding assays were performed using centrifugation as previously described.¹⁷ All solutions were prepared with 0.2 μ m-filtered PBS in 0.5 ml polypropylene tubes. Fluorescence binding was assayed in a 96-well plate format using a Biotek Synergy 4 plate reader (BioTek Instruments) equipped with a xenon flash bulb.

Saturation binding assays were used to determine the binding affinity (K_d) of the fluorescent 5/6-carboxyrhodamine 110-labeled PiB compound **4**. Briefly, 200 μ l solutions containing 10 μ g of A β (1–40) fibrils and various concentrations of compound (0–1000 nM, diluted 100-fold from DMSO stocks) were prepared in technical duplicate. Control solutions lacking synthetic A β (1–40) fibrils were likewise prepared to measure non-specific adsorption of the compound to the polypropylene tubes. After a 30 minute incubation at room temperature, the solutions were centrifuged at 16,000 $\times g$ for 15 minutes at room temperature. The resultant pellets were resuspended in 200 μ l of PBS and transferred to a black flat-bottom polypropylene 96-well plate (sc-204462, Santa Cruz Biotechnology). Fluorescence intensity

was measured using monochromators at 348 nm (5 nm bandwidth) excitation and 430 nm (5 nm bandwidth) emission. Specific binding to A β (1–40) fibrils was determined by subtracting the fluorescence intensity of the compound-only control solutions from those of the A β (1–40) plus compound solutions. Binding affinities were derived using the saturation binding one-site specific non-linear regression analysis model of GraphPad Prism 5 (GraphPad Software Inc., California).

Competition binding assays were used to determine the binding affinities of PiB and its clickable derivative 6-(propargyl-PEG₃)-BTA-1 (**3**) with aggregated A β (1–40). The fluorescently-labeled **4** was used as the reporter ligand at a constant concentration (140 nM), while the competitor ligands PiB (**2**) and clickable PiB (**3**) were used at concentrations ranging from 0 to 100,000 nM. Solutions were prepared in technical duplicate containing 10 μ g of aggregated A β (1–40), 140 nM of fluorescent clicked PiB (**4**), and the appropriate concentration of the competitor ligands (**2** and **3**). Control solutions lacking A β (1–40) were likewise prepared to measure the non-specific adsorption of the compounds to the polypropylene tubes. After a 30 minute incubation at room temperature, the solutions were centrifuged at $16,000 \times g$ for 15 minutes. The resultant pellets were resuspended in 200 μ l of PBS, and transferred to black flat-bottom, polypropylene 96-well plates. Fluorescence intensity was measured using a 485 nm (20 nm bandwidth) excitation filter and a 528 nm (20 nm bandwidth) emission filter (BioTek Instruments). Specific binding to A β (1–40) fibrils was determined by subtracting the fluorescence signals of the compound-only control solutions from those of the A β (1–40) plus compound solutions. Binding affinities were derived using the competitive binding one-site fit K_i non-linear regression analysis model of GraphPad Prism 5 (GraphPad Software Inc., California).

***In vitro* assays of compound binding with human brain extracts**

Single-concentration centrifugation binding assays were performed using the SDS-extract fractions from six control and six AD cases. Briefly, 100 μ g of the SDS-extracts were diluted to 200 μ l with TBS and incubated with 100 nM of the fluorescent clicked PiB compound (**4**) (diluted 100-fold from DMSO solutions) for 2 hours at room temperature with rotation. Control solutions were prepared with 100 μ g of the SDS-extracts and equivalent volumes of DMSO without the fluorescent compound. The samples were fractionated by ultracentrifugation at $150,000 \times g$ for 1 hour at 10TMC, and the pellets were resuspended in 200 μ l of PBS and transferred to black polypropylene 96-well plates. Fluorescence intensity of the resuspended pellets was measured using a 485 nm (20 nm bandwidth) excitation filter and a 528 nm (20 nm bandwidth) emission filter (BioTek Instruments). Binding of the fluorescent clicked PiB (**4**) compound to the brain extracts was determined by subtracting the background autofluorescence of the control solutions from the fluorescence signal of the brain extracts plus fluorescent clicked PiB solution. In all experiments, we elected not to chemically quench autofluorescence (mostly due to lipofuscin) in order to preclude potential interference with the PiB binding and/or signal. The adjusted fluorescence intensities were graphed, and the signals of the AD and control groups were statistically compared using Student's *t*-test with GraphPad Prism 5 (GraphPad Software Inc., California).

Staining of AD brain sections with fluorescently labeled clicked PiB (4) and anti-A β antibodies

Paraffin-embedded samples of frontal cortex from pathologically confirmed AD cases were obtained from the Emory Alzheimer's Disease Research Center (ADRC) brain bank, and 10 μ m-thick sections were stained with fluorescently-labeled clicked PiB (4). Briefly, slides were deparaffinized by heating at 60^oC on their sides for 60 minutes to remove the majority of paraffin wax, then sequentially immersed in xylenes (2 $\times$ 10 min), absolute EtOH (2 $\times$ 5 min), 95 % EtOH (2 $\times$ 5 min), 70% EtOH (1 $\times$ 5 min) and finally in PBS. Tissue sections were encircled with a PAP PEN (#71310, Electron Microscopy Sciences), allowed to dry, and incubated in darkness with 100 nM fluorescent clicked PiB in PBS for 60 minutes at room temperature. After rinsing for 5 seconds in PBS, the slides were coverslipped with DAKO Fluorescence Mounting Medium (S3023, Agilent Technologies) and images were immediately captured with a fluorescence microscope (Leica DMLB, Wetzlar Germany) using the FITC filter set (excitation filter: 460–495 nm, 505 nm beamsplitter, and emission filter: 515–560 nm).

After photographing the fluorescent clicked PiB staining, the slides were de-coverslipped and re-hydrated in preparation for immunohistochemical staining with antibody 6E10 to A β (1–16) (Covance, Princeton, NJ). The tissue was treated with 90% formic acid to expose antigenic sites on A β , rinsed in deionized water, then immersed in 1% H₂O₂ for 30 minutes, 0.3% Triton X-100 for 10 minutes and 5% blocking serum for 30 minutes, all in TBS. The tissue was subsequently incubated with antibody 6E10 diluted 1:5000 in TBS overnight at 4^oC. The following day, the sections were washed in TBS and the antigen-antibody complex detected using the avidin-biotin peroxidase method (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine (DAB) as the chromogen. Sections were dehydrated, re-coverslipped, and the same fields in which images of fluorescent clicked PiB staining had been captured were re-photographed at the same magnifications using bright-field illumination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Thioflavin T (1)

6-OH-BTA-1 (2)

(PiB)

6-(propargyl-PEG₃)-BTA-1 (3)

(Clickable PiB)

Figure 1. Several amyloid ligands based on the benzothiazole-aniline (BTA) scaffold (1–3)
Note the cationic heterocyclic amine of Thioflavin T (**1**). Removal of the benzothiazolium methyl group from **1** afforded charge-neutral ligands such as 6-OH-BTA-1 (PiB, **2**). The PEGylated “clickable” PiB derivative (**3**) was synthesized and characterized as described in this paper.

Figure 2. General scheme of the CuAAC click reaction used to generate multiple covalent conjugates from a single starting material

Clickable PiB derivative **3** was conjugated to an azide-labeled fluorophore to yield 5/6-carboxyrhodamine 110-labeled PiB (**4**) (left arrow). Likewise, clickable PiB (**3**) was conjugated to 1 μm diameter azide-coated superparamagnetic beads to yield the magnetic bead PiB conjugate **5** (right arrow).

Figure 3. Saturation and competitive fluorescence binding assays with synthetic A β (1–40) aggregates

(A) A saturation binding assay was used to derive a K_d of 164.7 nM for fluorescent clicked PiB (4). (B) Dose-response curve showing displacement of fluorescent clicked PiB (4) (at 140 nM) with increasing concentrations (0–100,000 nM) of PiB (2) (●) and clickable PiB (3) (▲). From these data, binding affinities (K_i) of 678.4 and 264.7 nM were determined for compounds 2 and 3, respectively (summarized in Table 1).

Figure 4. Fluorescent clicked PiB selectively binds to A β in AD brain

(A) AD brain sections were stained with 100 nM of fluorescent clicked PiB (**4**) (left panel). Immunohistochemistry was subsequently performed on the same section with the A β antibody 6E10, confirming that compound **4** selectively binds to A β plaques in AD brain. When imaged in the red channel (middle panel), the clicked PiB fluorescence signal disappears, leaving only the background autofluorescence of lipofuscin. The arrows mark the same regions in all three images, demonstrating colocalization of fluorescent clicked PiB staining and A β plaques. Scale Bars=50 μ m. (B) Another AD brain section stained with 100 nM of fluorescent clicked PiB and 6E10. The white arrows mark selected PiB-positive A β plaques. Note that the clicked fluorescent PiB strongly labels plaque cores, but diffuse A β deposits are either weakly positive or negative. Scale Bars=100 μ m. (C) Single-concentration binding assay with fluorescent clicked PiB (**4**) and 100 μ g of SDS-fractions from control (n=7) and AD (n=7) brains. The fluorescent PiB derivative detected a statistically significant (*p = 4.9×10^{-5}) difference in the degree of binding between control and AD brain extracts. P-value was determined using a two-tailed Student's *t*-test.

Figure 5. Affinity-capture of A β aggregates from AD brain with PiB-labeled magnetic beads (5) (A) Azide magnetic beads were covalently labeled with clickable PiB, and aliquots of the reaction supernatant were analyzed by fluorescence spectroscopy to determine the degree of bead functionalization (top panel). The fluorescence wavelengths corresponded to the intrinsic fluorescence spectrum of the PiB scaffold. As expected, a sham reaction without the copper sulfate catalyst maintained steady levels of free clickable PiB, while the fluorescence signal of the conjugation reaction decreased to ca. 10% of its initial value, indicating thorough bead labeling. (B) Representative dot blot of affinity pulldowns from AD brain extracts using the magnetic bead-PiB conjugate **5**. (C) Densitometry revealed a statistically significant enrichment of A β (* $p = 1.82 \times 10^{-6}$) in the PiB bead pulldowns ($n=6$) relative to bead controls ($n=6$). P-value was determined using a two-tailed Student's t -test.

Table 1Binding Constants of Compounds 2–4 with Aggregated A β (1–40) and Fluorescence Detection Wavelengths

Cmpd No.	Ex. λ (nm)	Em. λ (nm)	cK_d or dK_i (nM)
2	348 ^a	430 ^a	678.4 $\pm$ 1.1 ^d
3	348 ^a	430 ^a	264.7 $\pm$ 1.1 ^d
4	485 ^b	528 ^b	164.7 $\pm$ 11.02 ^c

Fluorescence excitation (Ex. λ) and emission (Em. λ) wavelengths were^a experimentally determined (2–3) or^b close to published values for the given fluorophore (4).^c K_d values were determined by saturation binding assays, and^d K_i values were determined by competition binding assays with fluorescently-labeled 4 as the reporter ligand.