Delineation of Positron Emission Tomography Imaging Agent Binding Sites on β-Amyloid Peptide Fibrils*

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A range of imaging agents for use in the positron emission tomography of Alzheimer’s disease is currently under development. Each of the main compound classes, derived from thioflavin T (PIB), Congo Red (BSB), and aminophenanthrene (FDDNP) are believed to bind to mutually exclusive sites on the β-amyloid (Aβ) peptide fibrils. We recently reported the presence of three classes of binding sites (BS1, BS2, BS3) on the Aβ fibrils. We have recently reported that the interaction of the thioflavin T probe (BS1 probe) indicated that both PIB and FDDNP were able to displace the radioligand with Ki values of 25 and 42 nM, respectively. BSB was unable to displace the radioligand tracer from the Aβ fibrils. In contrast, each of the compounds examined were able to displace thioflavin T (BS1 probe) from the Aβ fibrils when evaluated in a fluorescence competition assay with Ki values for PIB, FDDNP, and BSB of 1865, 335, and 600 nM, respectively. Finally, the Ki values for FDDNP and BSB binding to Aβ fibrils were directly determined by monitoring the increases in the ligand intrinsic fluorescence, which were 290 and 104 nM, respectively. The results from these assays demonstrate that (i) the three classes of thioflavin T binding sites are able to accommodate a wide range of chemotype structures, (ii) BSB binds to two sites on the Aβ fibrils, one of which is BS2, and the other is distinct from the thioflavin T derivative binding sites, and (iii) there is no independent binding site for the fibrils for FDDNP, and the ligand interacts with both the BS1 and BS3 sites with significantly lower affinities than previously reported.

The development of imaging agents to detect the senile plaques and neurofibrillary tangles associated with Alzheimer disease (AD) is a rapidly emerging and important field for both preclinical and clinical drug development (1). Three main structural classes or chemotypes of positron emission tomography imaging agents are currently under development and are derived from thioflavin T (PIB), Congo Red/styrylbenzene (ISB and BS3), Congo Red/styrylbenzene (ISB, BS5, X34 (6), stilbene (7)) and aminophenothiazine (FDDNP, FENE (8)) backbones. These ligands are primarily believed to target the polymeric form of β-amyloid (Aβ) peptide associated with the senile plaques and may in addition bind to neurofibrillary tangles, which are composed of tau protein polymers (1).

Because the number of compounds under development has increased, a somewhat confusing picture has emerged with respect to the interaction of members of the different chemotype classes with the Aβ fibrils. Initial findings, which were based on radioligand binding assays, indicated an independent binding site on the Aβ fibrils for each of the three chemotypes. For example, Agdeppa et al. (9) demonstrated that [35F]FDDNP was not displaced from Aβ fibrils by either Congo Red or thioflavin T. In addition, data from Zhuang et al. (4) indicated that the binding sites for TZDM and ISB were mutually exclusive. In contrast, data from Suemoto et al. (10), using a fluorescence assay format, indicated that both X34 and FDDNP were able to displace thioflavin T from Aβ fibrils. Finally, Kung et al. (11), using AD brain homogenates rather than in vitro generated Aβ fibrils, found that FDDNP could displace a radiolabeled stilbene derivative SB13.

We have recently reported that the interaction of the thioflavin T class of ligands is much more complex than previously appreciated with three classes of binding sites (BSs) present on in vitro generated Aβ(1–40) fibrils (12); they are BS1, a medium density site associated with the high fluorescence state of thioflavin T, BS2, a high density site with binding specificity for halogen-containing ligands such as TZPM and TZPI, and BS3, a low density site detected primarily by radioligand assays. In light of these findings we have investigated whether the apparently contradictory results of the displacement assays could be rationalized in terms of the three-site binding model we proposed for the thioflavin T ligands. We have, therefore, examined the interaction of selected compounds from each of the main imaging agent chemotypes with Aβ fibrils under a standard set of conditions using a range of binding assay formats. The results from this study extend our previous model of ligand binding sites on Aβ fibrils in that it demonstrates that BS1, BS2, and BS3 are able to accommodate a range of chemotype structures. Additionally, the results suggest that FDDNP does not have a single discrete type of binding site on the fibrils.

MATERIALS AND METHODS

Compound Names and Sources—Full chemical nomenclature for the ligands used in this study are as follows: 2-(4′-methylaminophenyl)-6-hydroxybenzothiazole (6-OH-BTA-1/PB) (2), 2-(1-[6-[(2-fluoroethyl)(methyl)amino]-2-naphthylethylidene)malononitrile (FDDNP) (8); (trans, trans)-1-bromo-2,5-bis-(3-hydroxybenzyl)-4-hydroxy-styrylbenzene.
benzene (BSB) (4). These compounds were custom-synthesized and confirmed for purity by reverse-phase high performance liquid chromatography, one-dimensional NMR, and mass spectrometer analysis. Thioflavin T was obtained from Merek, and (S)-naproxen ((S)-2-(6-methoxy-2-naphthyl)propionic acid) was obtained from Sigma. Radiolabeled 2-[4-\(^{3}H\)](methylamino)phenyl]-6-methylbenzothiazole ([\(^{3}H\)]Me-BTA-1) (84 Ci/mmol, 1 mCi/ml) was custom synthesized by Amersham Biosciences. The structures of these compounds are shown in Fig. 1.

**Polymerization of Aβ Fibrils—**Human Aβ-(1–40) peptide (Batch MK0611 from California Peptide Research, Napa, CA) was prepared in PB buffer (10 mM sodium phosphate, 1 mM EDTA, pH 7.4) and characterized as described previously (12).

**Compound Preparation—**All compounds were prepared as 10 mM MeSO stocks before dilution into assay buffer. The maximum final concentration of MeSO in the assays was 0.5%. Because of the relatively low solubility of the ligands (with the exception of thioflavin T) in aqueous solution, all assays were performed in PB supplemented with 10% ethanol.

**Radioligand Competition Assays—**Competition assays employed a fixed concentration of Aβ-(1–40) (40 nM) and [\(^{3}H\)]Me-BTA-1 (4 nM) and used varying concentrations ranges of cold competitor dependent on the ligand as described in Lockhart et al. (12). All data points were performed in triplicate, and the specific binding signal in the absence of competitor defined a fractional binding of 1. Data were analyzed in Grafit (Erithacus Software Ltd., Horley, UK) to obtain IC\(_{50}\) values using full four-parameter curve fits. K\(_i\) values were derived from the Cheng-Prusoff equation (13), K\(_i\) = IC\(_{50}\)/(1 + (L/K\(_i\)), where L was the concentration (4 nM); the K\(_i\) (3.25 nM) value of the radioligand was used in the assays.

**Fluorescence Competition Assays—**Competition assays employed a fixed concentration of Aβ-(1–40) (2 \(\mu\)M) and either thioflavin T (1 \(\mu\)M) or FDDNP (0.4 \(\mu\)M) as the fluorescence tracers and varying concentrations of competitor ligands. Reactions were performed in PB supplemented with 10% ethanol in a final volume of 80 \(\mu\)l and were incubated for 2 h at 20 °C before measurement in an Ultra Evolution plate reader (Tecan, Mannedorf, Switzerland) with filter pairs of either 450–505 nm (thioflavin T) or 340–440- or 450–535-nm filter pairs, respectively. The resulting intensities, corrected where necessary for background fluorescence (FLINT2), were plotted in the Ultra Evolution plate reader using either 340–440- or 450–535-nm filter pairs, respectively. The resulting intensities, corrected where necessary for background fluorescence (FLINT2), were plotted in the Ultra Evolution plate reader using either 340–440- or 450–535-nm filter pairs, respectively. The resulting intensities, corrected where necessary for background fluorescence (FLINT2), were plotted in the Ultra Evolution plate reader using either 340–440- or 450–535-nm filter pairs, respectively.

**Intrinsic Fluorescence Intensity Binding Assays—**Intrinsic fluorescence intensity (FLINT) changes associated with ligand binding to Aβ-(1–40) were performed as described previously (12). Briefly, FLINT1 was performed using a fixed concentration of ligand (25 or 100 nM) and varying concentrations of Aβ-(1–40) polymer. FLINT2 was performed using a fixed concentration of Aβ-(1–40) (500 nM) and varying concentrations of ligand (0 to 2 \(\mu\)M) as detailed under “Results.” The fluorescence signal from the BSB and FDDNP ligands were detected in the Ultra Evolution plate reader using either 340–440- or 450–535-nm filter pairs, respectively. The resulting intensities, corrected where necessary for background fluorescence (FLINT2), were plotted in the Ultra Evolution plate reader using either 340–440- or 450–535-nm filter pairs, respectively. The resulting intensities, corrected where necessary for background fluorescence (FLINT2), were plotted in the Ultra Evolution plate reader using either 340–440- or 450–535-nm filter pairs, respectively. The resulting intensities, corrected where necessary for background fluorescence (FLINT2), were plotted in the Ultra Evolution plate reader using either 340–440- or 450–535-nm filter pairs, respectively.

**RESULTS**

**Radioligand Competition Assays Probing BS3 Interactions—**Competition assays used the previously characterized compound [\(^{3}H\)]Me-BTA-1 which is a ligand for BS3 (12). The ligand had a K\(_i\) of 3.25 nM for this batch of Aβ fibrils (data not shown), which compares favorably with our previously published value of 4.2 nM (12). As expected, the thioflavin T derivative 6-OH-THP was able to completely displace the radioligand (RL) from the Aβ fibrils in a dose-dependent manner with a K\(_i\) value (K\(_i\)RL) of 25 nM (Fig. 2 and Table I). The K\(_i\) value reported here is higher than the previously reported K\(_i\) for PIB of 4.7 nM (2). The aminonaphthalene compound FDDNP was able to compete with [\(^{3}H\)]Me-BTA-1 in a dose-dependent manner (K\(_i\)FDDNP = 42 nM) and is in contrast to a previous study in which radiolabeled FDDNP was not displaced by either thioflavin T or Congo Red (9). The compound (S)-naproxen, which shares some structural similarities with FDDNP (Fig. 1) and had previously been demonstrated to displace [\(^{18}F\)]FDDNP from Aβ fibrils (9), was inactive over the range of concentrations used in this assay format.

The Congo Red derivative BSB did not display a significant dose-dependent displacement of the radiotracer from the Aβ fibrils. The fractional displacement of [\(^{3}H\)]Me-BTA-1 by BSB was more variable than any of the other ligands examined and showed a general trend toward higher values of radiotracer binding (i.e. a fractional binding greater than 1) at the highest concentrations of competitor, a phenomenon that has also been observed by Kung et al. (11). We have no clear explanation for this behavior, but it may be that the symmetrical nature of
BSB cross-links the fibrils, which in turn lead to a nonspecific trapping of radiotracer on the filters. Alternatively, BSB may induce a conformational change in the fibril structure that leads to an apparent increase in the binding capacity of the polymer for the radioligand. However, our data strongly suggests that BSB does not directly compete with [3H]Me-BTA-1 for BS3, and this conclusion is consistent with Zhuang et al. (4), who first demonstrated that the binding sites on Aβ fibrils for thioflavin T and Congo Red derivatives were discrete.

**Fluorescent Competition Assays Probing BS1 Interactions**—The initial set of assays was performed using thioflavin T as a tracer for BS1. All of the compounds assayed were able to completely displace the thioflavin T in a dose-dependent manner, consistent with displacement from a single population of binding sites (Fig. 3 and Table I). The $K_i$ values ($K_{i,FL-TT}$) for PIB, FDDNP, and BSB were 1865, 335, and 600 nM, respectively. (S)-Naproxen was only able to displace the thioflavin T at very high concentrations, and although no direct determination for this compound was attempted, its $K_{i,FL-TT}$ was estimated to be $>20 \mu M$.

The results of these assays were different from the findings in the radioligand assays in two important respects. First, the pattern of compound binding between the two formats was different in that, whereas PIB and FDDNP were able to displace both the radioligand and fluorescence tracers, BSB was only able to displace the fluorescence tracer. Second, the $K_{i,FL-TT}$ values were significantly higher (i.e. weaker binding) than those reported from the radioligand assays.

The ability of PIB to displace from both the BS1 and BS3 sites was anticipated from our previous work with similar compounds such as IMPY and BTA-1 (12). However, the finding that FDDNP was able to displace from both sites and BSB from only BS1 was unexpected and suggested that these binding pockets displayed a much broader specificity than previously described.

**Determination of Binding Constants for FDDNP and BSB Binding to Aβ Fibrils**—The binding of FDDNP and BSB to Aβ fibrils was directly measured by monitoring the intrinsic fluorescence of ligands using two different assay formats, FLINT1 and FLINT2. These assays either vary the concentration of Aβ fibrils with a fixed concentration of ligand (FLINT1) or vary the concentration of ligand with a fixed concentration of Aβ fibrils (FLINT2). The binding isotherms from these assays were all consistent with ligand binding to a single population of binding sites on the fibrils (Fig. 4). The $K_i$ values derived from the assays, $K_{i,FLINT1}$ and $K_{i,FLINT2}$ for FDDNP and BSB were 13860 ± 290 nM and 300 ± 104 nM, respectively (Table I). The ratio of $K_{i,FLINT1}/K_{i,FLINT2}$ is a measure of the binding site density on the Aβ fibrils (12) such that FDDNP has 1 binding site every ~45 Aβ monomers, whereas BSB has 1 binding site per ~3 Aβ monomers. PIB did not produce a significant increase in its intrinsic FLINT when incubated with Aβ fibrils. This is a similar finding to that noted previously for the structurally related compound BTA-1 (12).

**Fluorescence Competition Assays Utilizing FDDNP**—The similarity of the $K_{i,FL-TT}$ value with that of the $K_{i,FL-P}$ value for FDDNP suggested that same site, BS1, was detected by both methods. Further evidence supporting this finding came from competition assays using FDDNP as the fluorescent tracer (Fig. 5 and Table I). A $K_i$ value ($K_{i,FL-P}$) for PIB of 1365 nM was obtained for the displacement of FDDNP from the Aβ fibrils, which is similar to the $K_{i,FL-TT}$ value of 1865 nM obtained for PIB in the thioflavin T displacement assay. This finding supports the argument that the site detected by the different fluorescence tracers is identical (i.e. BS1) and distinct from BS3. (S)-Naproxen was a poor displacer of the fluorescence tracer as noted also in the thioflavin T competition assays. The $K_i$ value for this compound was not determined but was estimated to be $>20 \mu M$.

Significant contamination of the FDDNP emission signal was observed in this assay format from both thioflavin T (data not shown) and BSB. This was derived from the intrinsic fluorescence of these compounds bleeding through the emission filter. As a consequence, no $K_i$ values could be accurately determined for these compounds. A limited dataset of BSB concentrations that did not show significant fluorescence interference has been plotted on Fig. 5 and demonstrates that the ligand is clearly able to displace the FDDNP from the Aβ fibrils. Although we were not able to define a full displacement curve from these experiments, the shape of the curve is consistent with a $K_i$ of similar magnitude to that observed in the thioflavin T competition assays.

**DISCUSSION**

The development of imaging agents for detecting and quantifying senile plaque deposition in AD will be a key technology in both the preclinical and clinical evaluation of potential new therapies. Two of the agents, PIB and FDDNP, have already been used in human studies and show increased retention in AD patients in areas of the brain associated with deposition of senile plaques (14, 15). PIB appears to possess the more attractive profile, primarily due to its apparently higher specific signal (16). It is, therefore, of great importance that the binding properties of such ligands are fully understood to enable the correct selection of imaging agents and to aid in the interpretation of data collected from imaging studies. We have, therefore, tried to gain a fuller understanding of (i) potential inconsistencies in how members of the three main ligand chemotypes interact on the polymers and (ii) the interaction of these ligands with Aβ fibrils.

Data from the radioligand competition assays using [3H]Me-BTA-1 support the original observation by Zhuang et al. (4) of independent binding sites for thioflavin T and Congo Red derivatives, as we found that BSB did not compete with the radiotracer. This finding has also been replicated by Kung et al. (11), who used AD brain homogenates rather than in vitro produced Aβ fibrils. Combined, these findings provide strong support that the site detected by [3H]Me-BTA-1, BS3, does not bind Congo Red derivatives.

In contrast to previous reports for FDDNP (9), we found that the ligand was able to displace the radiotracer from BS3, consistent with a direct competition for this site. This finding is supported by the recent observation that FDDNP was able to specifically displace the radiolabeled ligand SB13 from AD brain homogenates (11). Although SB13 is a stilbene, it was
More complex series of calculations to determine, first, the free concentration of ligand (17) and then to use those values to determine the ratio of non-steroidal anti-inflammatory drugs such as (S)-naproxen and (S)-ibuprofen ((S)+(-)-2-(4-isobutylphenyl)propionic acid) to displace FDDNP from Aβ fibrils. Both of these structurally related drugs have been reported to displace [18F]FDDNP from Aβ fibrils with Ki values of 5.7 nM and 11 μM (9). In contrast, our data demonstrate that (S)-naproxen only competes with FDDNP at very high concentrations (K_{FPL-FD} > 20 μM), and in addition (S)-ibuprofen at concentrations of up to 100 μM is unable to displace any FDDNP from Aβ fibrils (data not shown). FDDNP and (S)-naproxen differ in their substituents at the 2 and 6 position of the napthyl ring (see Fig. 1) whereas (S)-ibuprofen and (S)-naproxen share an identical propionic acid group at the 2 position of their respective phenyl and napthyl rings. The apparent lack of activity in the displacement assay of (S)-ibuprofen and the weak activity of (S)-naproxen strongly suggest that the 2 position does not significantly contribute to binding specificity or affinity and that the fluoroethyl-substituted amino group at position 6 of the napthyl ring is the major determinant of ligand binding for this class of imaging agents.

The pattern of BSB binding to the Aβ fibrils in the fluorescence assays was also consistent with our previously published model of ligand binding sites. The ratio \( \frac{K_{FPL1}}{K_{FPL2}} \) for this ligand was ~3, which is consistent with that observed for ligands which interact with BS2, such as TZDM, TZPI, and BF1 (12). These latter three compounds all contain a halogen substituent on their aromatic ring systems, and this feature we believe determines their binding specificity for this site. Despite the different backbone structure of BSB compared with the halogenated thioflavin T derivatives, the Congo Red derivative also contains a bromide atom directly conjugated to one of its aromatic rings. The importance of the positioning of the halogen group is evinced through the observation that, although it has one, the halogen substituent of FDDNP is not directly conjugated to the aromatic ring system, and hence, the ligand does not show the same binding pattern as that observed for TZDM, TZPI, and BF1 (or BSB).

BSB does, however, differ slightly in its ability to displace thioflavin T compared with TZDM, TZPI, and BF1. These halo-
gen-substituted thioflavin T derivatives were found to displace around half of the fluorescent tracer (12), whereas BSB displaces almost fully. The ability of BSB to displace more of the tracer may be due to the significantly greater size of the molecule. Evidence from our previous work indicated that BS2 ligands could not only displace compounds from BS1 but that there was also a FRET signal between the sites, indicating that a significant proportion of the sites were also in close proximity to each other (12). It may, therefore, be that the greater molecular size of BSB fully occludes the BS1 sites when bound to the fibrils.

In conclusion, the data from this study have established that BS1, BS2, and BS3 are able to bind a wider range of compound structures than previously realized. This finding resolves the issue surrounding the ability of different chemotype classes to displace each other from Aβ fibrils. Second, the data questions the presence of a unique class of binding sites for aminonaphthalene compounds such as FDDNP by identifying both BS1 and BS3 as the major sites of interaction on the Aβ fibril. These results demonstrate that a thorough analysis of in vitro data is an important prerequisite in the selection of compounds for in vivo studies.

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