DOI: 10.1002/cmdc.200700188

Thioflavin Derivatives as Markers for Amyloid-β Fibrils: Insights into Structural Features Important for High-Affinity Binding

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Alzheimer's disease (AD) is a common neurological disease associated with chronic dementia, memory loss, and cognitive impairment. Central to the neuropathology of AD are the senile plaques (SPs) and neurofibrillar tangles (NFTs), depositions composed of amyloid-β peptide (Aβ) and tau protein, respectively.[1] Aggregated Aβ in senile plaques has a β-sheet secondary structure and is arranged as fibrils.[2,3] Soluble Aβ prior to aggregation is predominantly unstructured. Formation and accumulation of aggregates of Aβ peptides in the brain are critical factors in the development and progression of AD,[4,5] in which oxidative stress represents a field of current intensive studies.[6–8]

There is a great interest in molecules able to bind specifically to Aβ aggregates.[9,10] Such markers of Aβ fibrils would allow their early detection and specificity would permit identification of Aβ fibrils from other amyloid deposits. This is of importance from basic research to clinical application. In particular, such molecules give access to molecular imaging (by Single Photon Emission Computed Tomography (SPECT), Positron Emission Tomography (PET), or Magnetic Resonance Imaging (MRI)) therefore allowing a precise localisation and identification of the Aβ aggregates in the brain. This is in contrast with the less specific information obtained either by cerebrospinal fluid analysis,[11,12] or anatomic imaging by MRI.[13] The progress of therapies that may affect Aβ deposition in AD brain has added new significance to this pursuit.[14–16] Three main categories of PET ligands of AD-associated aggregates are currently under investigation (Figure 1): Thioflavin T (ThT) derived compounds (for example, *N*-methyl-[(11)*C*]2-(4′-methylaminophenyl)-6-hydroxybenzothiazole (PIB)), including variations of the heteroaromatic core, styrylbenzene (SB), and compounds with an aminonaphthyl core (for example, 2-(1-{6-[(2-[*F*-18]fluoroethyl)-(methyl)amino]-2-naphthyl}ethylidene)malononitrile (FDDNP)).

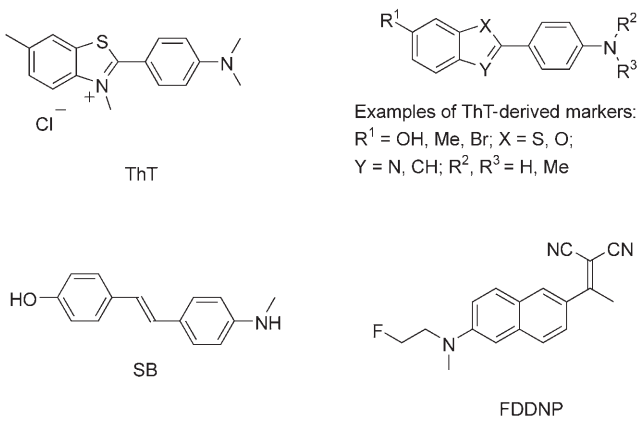

Figure 1. Structures of some known Aβ aggregate markers.

ThT has been used for several decades to stain amyloids such as Aβ. ThT is not specific for amyloids of Aβ, as it reacts with many types of amyloids (for example, NFT, insulin, β2-microglobulin) but not all.[17] Thus several derivatives of ThT have been generated with the aim of developing a biomarker of Aβ fibrils with high affinity and high specificity, as exemplified by BTA-1, *N*-methyl-^{11}*C*-PIB, ^{125}*I*-TZDM, etc.[18] Yet not much is known about the structure–function relationships that explain the molecular nature of the interaction marker/Aβ aggregate and most improvement of binding-affinity seemed to arise from empirical studies. The reported insights are scarce.[19] It seems clear that the removal of the methyl group on the heterocyclic nitrogen of ThT and hence the removal of the positive charge increases the affinity to Aβ fibrils by a factor of ≈40. Simultaneously, the removal of the charge increases the lipophilicity of the compounds and therefore eases crossing of the blood–brain barrier (BBB).[20] Also removal of one of the two methyl groups on the amine nitrogen in the 4′-position did increase the affinity for a so far unknown reason.[20,21] In contrast removal of the methyl group on the carbon 6 of the benzothiazole moiety did not significantly change the affinity.[22] Better knowledge of the binding sites of the markers derived from ThT would allow a more rational ligand design. To address this question and to get a deeper insight into the nature of the interaction between uncharged ThT derivatives and in particular the role of the amine nitrogen in the 4′-position, we synthesized 18 ThT derivatives of the dimethylaminophenyl moiety and compared their binding affinity to Aβ fibrils, which allowed us to propose some important features regarding the marker/Aβ fibrils interaction.

In the present work a highly convergent synthesis of ThT derivatives applied to 18 examples based on two similar reactions has been established (Scheme 1). Namely, the condensation of

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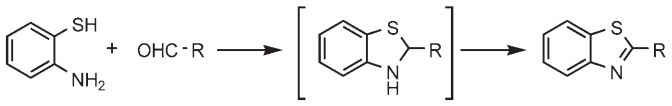

Scheme 1. General convergent synthesis of the target benzothiazoles.

2-aminothiophenol with an aldehyde, followed by spontaneous oxidation of the intermediate saturated heterocycle by atmospheric oxygen. This strategy compares well with the most recent advances in the synthesis of 2-aryl benzothiazoles bypassing multistep strategies, based on palladium catalysed coupling and involving starting materials such as 2-bromobenzothiazoles,[23] benzothiazoles,[24] or 2-thio substituted benzothiazole.[25,26] Interestingly, the present work is carried out with commercially available starting materials under easily reproducible conditions and without manipulation under inert atmosphere.

Of the two reactions, one is performed in solution and catalysed by a lanthanide based Lewis acid; the other on silica under microwave irradiation. The efficiency of the two methods proved to be equivalent for aromatic aldehydes and aromatic heterocyclic aldehydes (with yields varying from 70 to 98%). The reaction in solution is conveniently carried out with an excess of aldehyde whereas on solid support an excess of aminothiophenol is used. The availability of both methods can be advantageous when it comes to the availability of the starting materials for purification purposes.[27] This new convergent method leading to the benzothiazole core of ThT is a significant improvement compared to the classical methods,[28] and led to a significant array of ThT analogues (Figure 2) offering many structural variations.

The apparent dissociation constant of compounds **1**–**18** were determined by fluorescence measurements according to Lockhart et al.[21] These measurements consist either of a direct measurement (K_d) (compounds which revealed a significant fluorescence change upon interaction with the fibrils) or indirect by competition with ThT (K_i). The deduced affinities are shown in Table 1.

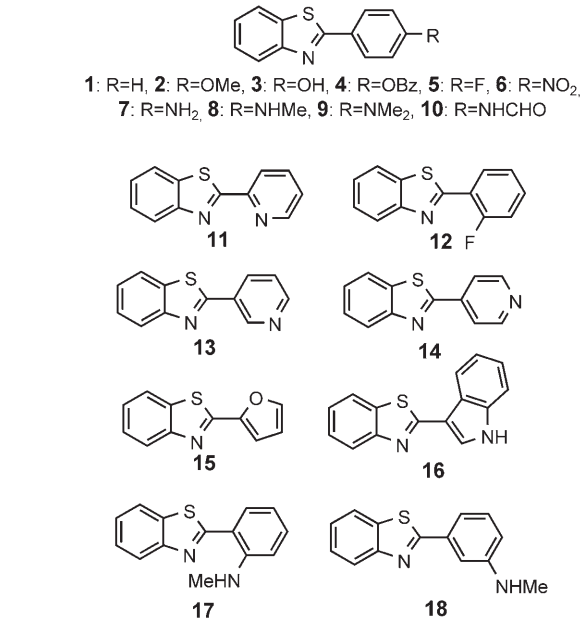

Figure 2. Compounds synthesised and evaluated.

Table 1. Affinities of the synthetic compounds toward fibrils of Aβ 1–40.

Compd	K_d [μM]	K_i [μM]
ThT	0.79 ± 0.05	–
4	–	1.65 ± 0.30
1	0.99 ± 0.01	1.3 ± 0.3
6	0.82 ± 0.09	1.15 ± 0.30
2	–	0.28 ± 0.07
12	0.30 ± 0.03	0.38 ± 0.08
5	0.28 ± 0.03	0.24 ± 0.05
13	–	0.31 ± 0.08
11	–	0.33 ± 0.08
14	–	0.21 ± 0.05
9	0.14 ± 0.03	0.24 ± 0.06
15	–	0.21 ± 0.04
16	–	0.032 ± 0.010
3	–	0.027 ± 0.005
10	–	0.33 ± 0.06
7	–	0.034 ± 0.005
8	0.0027 ± 0.0002	–
17	–	0.07 ± 0.01
18	–	0.017 ± 0.005

From these results a structure–affinity relationship study was engaged in which particular attention was paid to structural components able to improve the affinity of the derivatives of ThT, which is of potential interest for the design of high-affinity markers for Aβ fibrils.

All the derivatives were synthesised without the methyl-group on the nitrogen of the thiazole. As such all the components were uncharged, reported to have a higher affinity, and readily enter the brain.[20] In agreement with the literature, the removal of the methyl group on the heterocyclic nitrogen (compound **1**) led to a four- or fivefold increase of the affinity to Aβ fibrils.[20–22] This is likely due to the removal of the charge and thus a stronger interaction with the supposed hydrophobic binding pocket of the fibrils (a hydrogen bond with the lone pair of the intracyclic nitrogen might be postulated but it seems of moderate potency as benzothiophene and benzofuran derivatives have been shown to bind strongly to Aβ fibrils[29,30]).

The most prominent structural feature concerning the binding affinity was the presence of a X-H on the 2-aryl ring. All compounds (except **10**) featuring a X-H had high affinities with a $K_{d/i}$ below 100 nM, whereas all compounds without that moiety had a $K_{d/i}$ above 100 nM. Accordingly this could be interpreted as formation of a hydrogen bond between X-H and the fibrils leading to an increased affinity. Initially, we measured the affinities for compounds **7**, **8,** and **9** (that is. R-NH_2, R-NH-CH_3, and R-N$(CH_3)_2$), and found that the dimethylated nitrogen had a $K_{d/i}$ clearly above 100 nM, but the removal of one or both methyls improved the affinity to 3 and 30 nM, respectively. The *para N*-methylated aniline derivative showed the high-

est affinity (≈3 nM), almost an order of magnitude higher than all the other compounds. Although the higher affinity of the R-NH_2 and R-NH-CH_3 (compared to R-N(CH_3)$_2$) has been reported in the literature, no rationale has been developed.[20–21] We addressed the question by replacing the nitrogen with an oxygen. Whereas R-O-H (compound **3**) has a K_d of ≈30 nM, the affinity dropped by an order of magnitude when OH was exchanged with OMe (compound **2**) or even more with a bulky ester OBz (compound **4**). This indicated that a hydrogen donor (N-H or O-H) could be responsible for the elevated binding of R-NH_2/R-NH-CH_3 compared to R-N(CH_3)$_2$. The reason why the NH-CH_3 is stronger than NH_2 is not clear as the ability of the nitrogen to act as a H-bond donor is decreased by substitution (the strength of hydrogen bond depends on the same factors that determine the ability of a H donor to act as an acid). It could nevertheless be postulated that an additional hydrophobic interaction between CH_3 and the fibrils could be involved. This would favour the required N–H···X alignment for optimal H bonding and also explains the lower affinity of **3** compared to **8** (Figure 3). The case of compound **10** could be explained in terms of bulk strain, but is even more likely due to the lack of hydrophobicity (compared to a methyl) induced by the formyl adduct.

Moreover moving the NH-CH_3 group around the aromatic ring leads to a substantial but not drastic loss of affinity. A K_i of 17 nM and 70 nM were determined for NH-CH_3 in the *meta* and *ortho* position, respectively. It is conceivable that the same H-bond acceptor is involved for NH-CH_3 in the *meta* and *para* positions, but the hydrophobic interaction of the CH_3 with the same hydrophobic site seems less liable. In the case of the *ortho*, neither interaction can be maintained. Although a significant drop of the affinity was observed, the K_i was still below 100 nM. Thus it seems that the binding site can accommodate important structural alterations or more probably that it bears other groups allowing the formation of a hydrogen bond close to a hydrophobic pocket. In theory NH-Me can not only act as a H donor as evidenced above (Figure 3), but also as a H acceptor (by the way of its lone pair; Figure 3 dashed line). A general trend in the analyses of some of the 18 ThT derivatives seems to indicate that such an interaction may modestly contribute. Indeed compounds **2**, **5**, **9**, **11**, **12**, **13**, **14**, and **15** (all bearing a H-acceptor functionalities) exhibit affinities in the same range, that is, three- to fourfold lower than that of **1**.

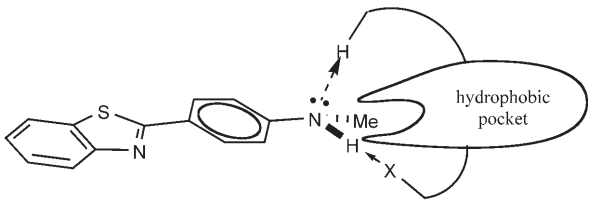

Figure 3. Explaining the higher affinity of compound **8** with the Aβ fibrils. The dashed lines indicate a potential H donor site.

Introduction of a methoxy in the *para* position, fluoro in the *ortho* or the *para* positions, and the change from an aryl ring to a pyridine ring (with benzothiazolyl moiety at position 2, 3, and 4) or 2-furan did not dramatically change the affinity (all the K_d were between 0.2 and 0.4 μM). This indicates that the electron density of the aromatic ring does not have a major effect on the affinity and therefore almost rules out any interactions of π type (π stacking, H–π interactions, etc.) between this aromatic ring of the ThT derivatives and the Aβ fibrils.

In conclusion, we have set up a new and highly convergent strategy giving access to compounds bearing the benzothiazole structure of ThT reported to be readily taken up in the brain, a prerequisite for in vivo analysis. Structural requirements for the relatively high affinity of compounds derived from ThT have been rationalised. Developments are currently under progress in order to assess the influences on the affinity toward Aβ aggregates of the exocyclic nitrogen substituent (in terms of bulk and hydrophobicity), of the benzothiazole ring, and of the substitution of the latter.

Acknowledgements

The authors thank the Association France Alzheimer for a student fellowship to R.L.Y., the CNRS (ATIP) and the Université Paul Sabatier (BQR ASUPS A03) for financial support.

Keywords: amyloid β · convergent synthesis · heterocycles · peptides · structure–activity relationships

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Received: July 12, 2007
Revised: September 10, 2007
Published online on October 9, 2007

