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Proflavine derivatives as fluorescent imaging agents of amyloid deposits

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ABSTRACT

A series of proflavine derivatives for use to further image Aβ amyloid deposits were synthesized and characterized. Aged 3xTg-AD (23 months old) mice hippocampus sections incubated with these derivatives revealed preferential labeling of amyloid plaques. Furthermore an in vitro binding study showed an inhibitory effect, although moderate, of these compounds on $A\beta_{40}$ fibril formation. This study highlights the potential of proflavine as a molecular scaffold for designing new Aβ imaging agents, its native fluorescence allowing in vitro neuropathological staining in AD damaged brain sections.

The extracellular deposits of β-amyloid peptide (Aβ) in senile plaques and intracellular neurofibrillary tangles (NTF) are known histopathological hallmarks of Alzheimer's disease (AD) in brain areas essential for cognitive functions. Especially, the accumulation of Aβ fibrils in plaques is believed to be fundamental to the initial development of the disease and to trigger a cascade of events such as neurotoxicity, oxidative damage, and inflammation leading to neuronal dysfunction and death.[1] Therefore, molecules that specifically bind β-amyloid aggregates might give access to AD's molecular imaging. Thioflavin T, Congo Red, stilbene and aminonaphthalene derivatives have been identified as markers and are under investigation for in vivo β-amyloid plaque imaging by positron emission tomography (PET).[2,3] A [^{18}F]fluoroacridine orange analogue with affinity for Aβ aggregates and amyloid plaques has also been identified.[4] To date, it is the first example of acridine derivatives developed as a marker although, as in most of the reported markers, its aromatic structure appears suitable to interact with the β-sheet rich Aβ fibrils (fAβ).

We propose here to expand the search for Aβ amyloid markers to other aminoacridine derivatives. We synthesized proflavine derivatives **1a–d** and **2a–d** (Fig. 1) and studied their ability to function as histological dyes to bind Aβ amyloid plaques from transgenic mouse in vitro.

The intrinsic fluorescence of the acridine portion allows the direct observation of the binding. The azide and alkyne derivatives **1b–d** are also suitable 'clickable derivatives' to allow a further radiolabeling for in vivo imaging using click methodology, especially with [^{11}C]methylazide and [^{18}F]fluoroalkynes, or Staudinger ligation.[5–7]

We also evaluated the interaction of these compounds with β-amyloid aggregates in vitro during the fibril formation process. Indeed, the acridine moiety of our derivatives may interfere with this process through hydrophobic and π-stacking interactions.[8] In hybrids **2a–d**, by adding lipoic acid or Tröger's base, we expect a synergetic effect and thus a better interaction with β-amyloid species. Lipoic acid, anti-oxidative agent with diverse pharmacologic properties in AD, is known to inhibit fAβ formation,[9,10] whilst the concave shape exhibited by Tröger's base analogues (Fig. 2) may be expected to interact within the grooves created by beta-sheets.

All acridine derivatives were prepared from 3-acetylamino-6-aminoacridine and most of them were obtained by simple precipitation (Scheme 1). The substitution of propargyl chloroformiate allowed the preparation of **1c** in one reaction step with 75% yield. Additional steps were required to introduce the amino free, azide or alkyne spacer arms of the other derivatives **1a**, **1b** and **1d**. Nevertheless, **1b** and **1d** were obtained in efficient yield of 75% and 65%, respectively. In the case of **1a**, the low isolated yield of 30% can be attributed to loss of materials during the purification steps by precipitation.

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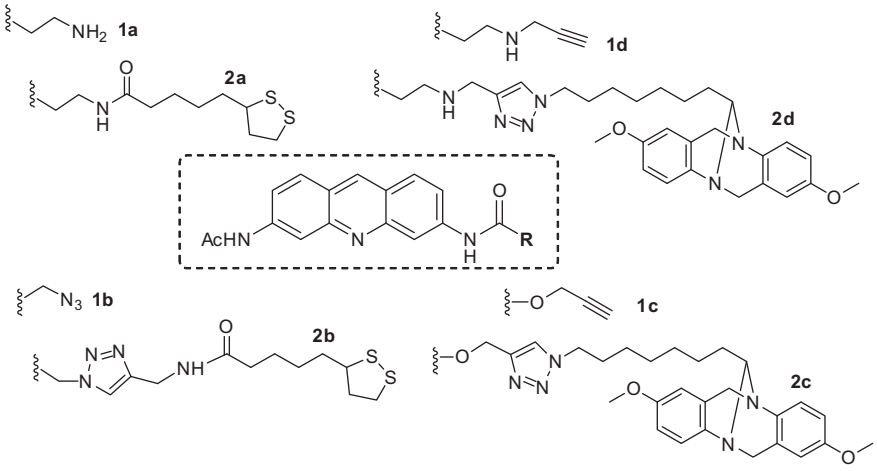


Figure 1. Structure of proflavine derivatives examined in this study.

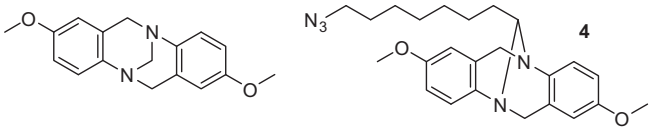
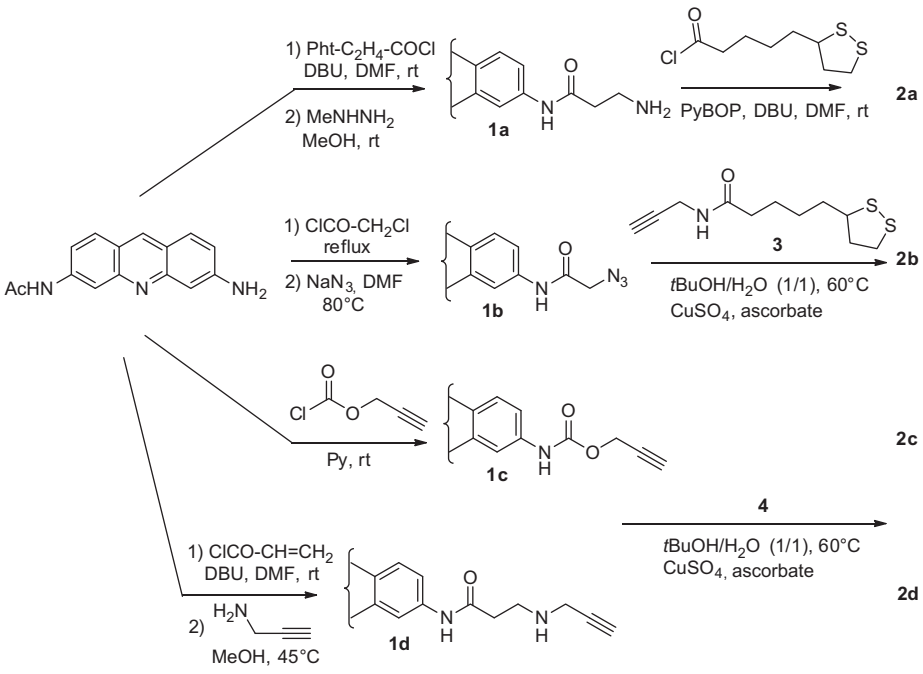


Figure 2. Tröger's base analogues.

The acridine heterodimers made from lipoic acid or Tröger's base **2a–d** were prepared from **1a–d** by ligation via an amide or triazole bond using copper catalyzed azide–alkyne cycloaddition. The common procedure using copper(II) sulfate and sodium ascorbate as reducing agent in *t*butanol/water solution proved to be efficient and the hybrids **2b–d** were obtained with good yields, 70–75%, from the appropriate alkyne or azide derivatives **3** and **4**. It should be noticed that the lipoyl derivative **3** and the Tröger's base analogue **4** were both produced as racemic mixture. Only a single enantiomer is shown for the Tröger's base derivatives.

The ability of compounds **1a–d** and **2a–d** to inhibit fAβ_{40} formation and to destabilize preformed fibrils was studied in vitro using thioflavin T (ThT) assays. The specific binding of ThT to amyloid fibrils is generally followed by spectroscopy, and this is the common way to identify amyloid structures, and to follow their formation and disaggregation. The compounds were first co-incubated for eight days at 10 μM with Aβ_{40} (50 μM) in the presence of ThT and the fluorescence change of the dye at 485 nm was measured. Results (Fig. 3) are expressed in percent compared to the fluorescence of control assay with Aβ_{40} alone.

The inhibitory activity of all tested acridine derivatives is relatively moderate compared to reference inhibitors, especially curcumin. Curcumin is a better inhibitor of fAβ formation than lipoic acid.[11] It is also known to bind amyloid plaques in vitro and in vivo.[12,13] Where curcumin showed an inhibition of 80% at 10 μM similar to those reported in the literature, our derivatives are typically around 50–60% of inhibition, with the exception of **1c** which is found slightly better inhibitor compared to the others.



Scheme 1. Synthesis of proflavine derivatives **1a–d** and **2a–d**.

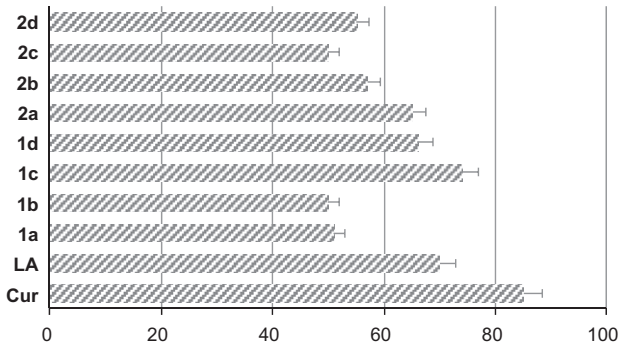


Figure 3. Inhibition % of $A\beta_{40}$ fibril formation. Lipoic acid (LA) and curcumin (Cur) are used as references. The data are the results of three independent experiments.

Moreover no benefit of the Tröger's base or lipoyl moiety in hybrids **2a–d** is revealed as compared with their acridine parents **1a–d**. On the contrary, the acridine core is found to be essential in hybrids since analogs of Tröger's base and lipoic acid, compounds **3** and **4**, are totally inactive as inhibitor of fAβ formation, even at a higher concentration of 50 μM. Whilst our derivatives are certainly not among the most potent inhibitors, it should be nevertheless noted that inhibition occurs at 0.2/1 molar ratio of test compound/$A\beta_{40}$ where other reported inhibitors, especially small peptide inhibitors, require higher excess of Aβ.

Regarding the fibril-destabilizing effects, none of our acridine candidates are able to disassemble preformed fibrils in vitro. Indeed the fluorescence of ThT remains unchanged after the incubation of fresh $fA\beta_{40}$ at 37 °C with our derivatives even at high compound/Aβ ratio of 1/2, conditions under which curcumin and lipoic acid are reported to disassemble 90% and 60% of fibrils, respectively.[10,11] This ThT assay also reveals that our derivatives are not able to inhibit ThT binding to synthetic $A\beta_{40}$ fibrils as it is the case for the fluoroacridine orange probe BF108[4] reported in the literature. Nevertheless, the ability of a compound to disaggregate fibrils or to displace the ThT probe is not to be related to its capability to bind or not bind fibrils. Indeed a lot of compounds bind fibrils in vitro without disaggregating them and it may be rather advantageous for markers. Moreover, the presence of several binding sites for probes, distinct from those of ThT, has been reported on Aβ peptide fibrils.[14]

The in vitro assembled Aβ fibrils are commonly used to screen the potential of probes to further bind amyloid plaques. However, we chose to directly investigate the ability of our candidates, **1a–d** and **2a–d**, to label amyloid β deposits from AD transgenic mice, which are more representative of those found within human senile plaques.

We used triple-transgenic mice (3xTg-AD)[15] harboring presenilin-1 ($PS1_{M146V}$), amyloid–beta precursor protein (APP_{swe}) and tau (tau_{P301L}) transgene. Brain frozen sections were prepared and incubated for 90 min with 100 μM compounds in buffer solution. Native fluorescence of acridine derivatives is exploited to reveal labeling of amyloid deposits, using fluorescein optics. Labeled plaques are confirmed by staining of the adjacent sections with thioflavin S, a pathological dye known to bind to Aβ plaques in the brain.[16] The results are illustrated in Figure 4 for compound **2d**. Twenty-three month aged 3xTg-AD mice hippocampus sections incubated with **2d** revealed labeling of dense core large plaques (Fig. 4A and B), while only a slight green auto-fluorescence ponctuated labeling is observed onto untreated 3xTg-AD brain slice in the region of amyloid deposit (Fig. 4C). No labeling is observed in age matched wild type mice (Fig. 4D). Finally, DAPI labeling does not recover the green labeling of amyloid plaques, excluding possible binding of the proflavine derivatives to DNA waste, which may be present in amyloid deposits.

As for **2d**, all other tested compounds clearly stained endogenous plaques of amyloid in the hippocampus of aged 3xTg-AD mice but with more or less efficiency. Indeed, **2d** with **1d** are the most efficient compounds related to the amount of labeled plaques, **1c** and **2c** are less efficient markers while **1a**, **2a** and **2b** present an intermediate behavior to label amyloid deposits. These results indicate no relationship between the structure of derivatives and their labeling efficiency, nor any correlation between their ability to bind plaques and to inhibit fAβ formation (Fig. 3). Moreover,

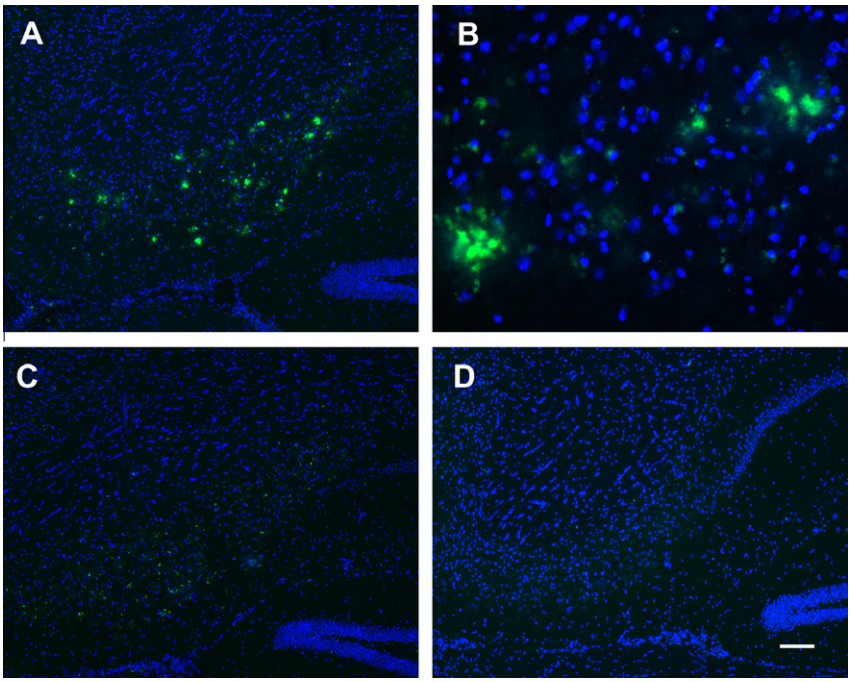


Figure 4. Medial aspect of the dorsal hippocampus, coronal sections from 3xTg-AD mouse brain incubated in the presence (A, B) or not (C) of compound **2d** at 100 μM. (D) Wild type mice brain section incubated with compound **2d** 100 μM. Scale bar in A, C, D = 100 μm and in B = 30 μm.

as already observed during the interaction studies with fAβ_{40} formation, no benefit of hybrid structures is revealed as regards the labeling ability.

In conclusion, we showed in this study the ability of some proflavine derivatives to interact in vitro with β-amyloid aggregates as well as with amyloid deposits in brain sections from AD transgenic mice. Moreover, this work highlighted the labeling potential of simple proflavine derivatives such as compound **1d**. Compared to the fluoroacridine BF108[4] which is reported as marker of amyloid plaques, the acridine derivatives tested in this study present similar labeling patterns. It appears, nevertheless, difficult from this preliminary study to compare their efficiency with this analogue since experimental labeling conditions used are different. Further studies are essential to determine if our candidates retain their labeling ability in human brain sections of AD patients and in vivo in AD transgenic mice models. Nevertheless the present study proves the potential of proflavine as molecular scaffold to design imaging probes of amyloid deposits, its native fluorescence being a real benefit for a first screening.

Acknowledgments

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Supplementary data

Supplementary data (the synthesis of compounds **2a**–**d** and the in vitro study of their interaction with Aβ_{40} aggregates and amyloid deposits) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.03.010.

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