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Disassembly of preformed amyloid beta fibrils by small organofluorine molecules

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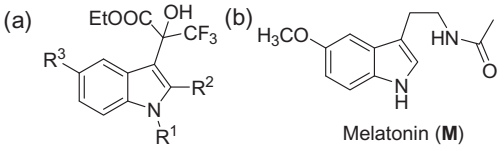
ABSTRACT

A potential therapeutic approach for Alzheimer's disease is to reduce the amount of toxic amyloid-beta oligomers and fibrillar amyloid plaques. In order to contribute to this approach the ability of small organofluorine compounds that were previously reported as successful inhibitors of fibrillogenesis to destabilize preformed fibrils of the amyloid-beta peptide was studied. These organofluorine molecules including chiral compounds were tested in vitro using standard methods based on Thioflavin-T (THT) fluorescence spectroscopy, atomic force microscopy (AFM) and Fourier-transform infrared spectroscopy (FTIR). It was observed that 5′-halogen substituted 3,3,3-trifluoromethyl-2-hydroxyl-(indol-3-yl)-propionic acid esters showed significant activity in the disassembly of the preformed fibrils. Since the same compounds were identified as strong fibrillogenesis inhibitors as well, this dual action makes them promising candidates for further drug development.

Alzheimer's disease (AD) involves misfolding and aggregation of the amyloid-beta peptide (Aβ) and its deposition into characteristic fibrils called amyloid plaques.[1] In the traditional amyloid cascade hypothesis of AD these fibrils are associated with pathology; while recent studies also emphasize the highly neurotoxic nature of precursor oligomers, which, in fact, are suggested being the most important link to neurotoxicity.[2–4] Therefore, the inhibition of the seeding and progress of Aβ self-assembly, complimented by the disassembly of the preformed aggregates is among the promising therapeutic options. One of the most pursued strategies is the inhibition of Aβ oligomerization and/or fibrillization by small organic molecules.[5,6] Some of these studies were also extended to the reverse process; the destabilization of the preformed fibrillar aggregates.[5–8] The modes of action of these molecules are still unclear, mostly due to the limited structural information available on the diverse amyloid assemblies.[9,10] The structure–activity relationship studies using strategic chemical modifications of the effectors can reveal important interactions and conformational requirements between ligands and peptides and also between the associating peptide units. Therefore, these studies can significantly contribute to the general understanding of amyloid formation and lead to a more rational design of new drug candidates.[11,12]



Compound	R^1	R^2	R^3
1	H	H	H
2	H	H	CH_3
3	H	CH_3	CH_3
4	CH_3	CH_3	H
5	H	CH_3	COOMe
6	H	CH_3	OMe
7	H	H	Cl
8	H	H	Br
9	H	H	I

Figure 1. (a) Organofluorine molecules used in the disassembly studies. (b) Melatonin, a well-known amyloid inhibitor, was also studied for comparison.

Continuing our earlier efforts on designing novel inhibitors of Aβ self-assembly, herein we describe the ability of small organofluorine inhibitors to act as disassembly agents. We have found that a number of trifluoromethyl-hydroxyl-(indol-3-yl)-propionic acid ethyl esters strongly inhibited the in vitro formation of Aβ fibrils.[13,14] In this Letter, we extend our above studies and determine the effect of these inhibitors on the stability of preformed fibrils. The main goal is to identify compounds that are not only

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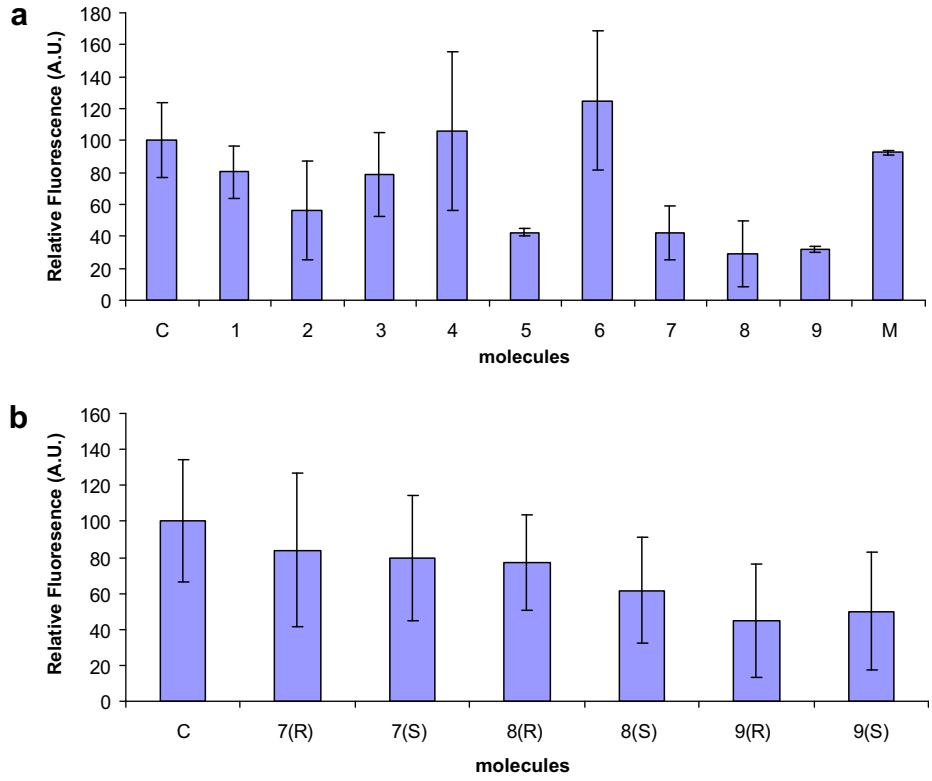

Figure 3. Relative THT fluorescence in the presence of (a) racemic trifluoro-hydroxyl-(indol-3-yl)-propionic acid esters and melatonin; (b) chiral 5′-halo derivatives of trifluoro-hydroxyl-(indol-3-yl)-propionic acid esters at 10:1 (small molecule:Aβ) molar ratio. The measured fluorescence intensities (I_{THT}) were normalized to the control sample C containing Aβ only (relative fluorescence: 100%). The data are average of three parallel experiments for (a) and four parallel experiments for (b), the values represent means ± standard deviation.

able to inhibit, but also to reverse the fibril formation. The chemical structure of the organofluorine compounds that have been applied in this study are shown in Figure 1 together with melatonin, a well-known amyloid inhibitor,[15] used for comparison.

The proposed compounds were synthesized and obtained in excellent yields and selectivities using procedures described previously.[16] In addition to the racemic compounds, three enantiomer pairs have been synthesized, as chiral compounds may have significantly different effects on biomolecules. The structures of the chiral molecules tested are shown in Figure 2.

For the disassembly experiments the fibrils were grown following the same protocol as described earlier.[13,14,17,‡] The data obtained after 4 days of incubation are shown in Figure 3. They clearly indicate the ability of these racemic as well as enantiomeric organofluorine molecules to disassemble the preformed fibrils although to different extent. The best results were obtained with the iodo-derivatives **9**, **9**(*R*), **9**(*S*). Furthermore, only negligible differences were found for the enantiomeric pairs indicating little or no role of chirality in the disassembly. Both of these results are similar to our earlier findings in case of fibril inhibition.[14]

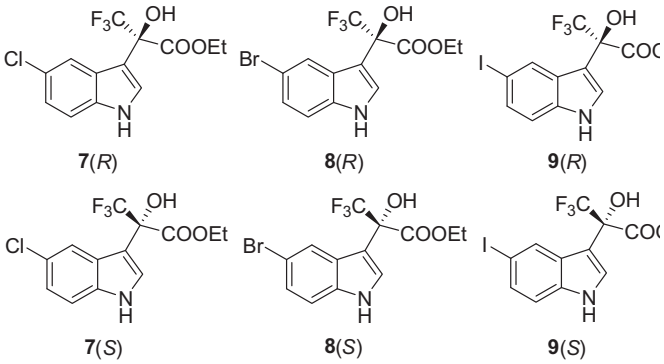

Figure 2. Structure of the enantiomeric trifluoro-hydroxy-(indol-3yl)-propionic acid esters.

Morphological characterization of the samples was carried out by Atomic Force Microscopy, using a Quesant Q-Scope 250 microscope in non-contact mode following the procedure described previously.[14,18,§] The images are shown in Figure 4.

‡ The synthetic lyophilized $A\beta_{1-40}$ peptide was dissolved in 100 mM NaOH to a concentration of 40 mg/ml and diluted in 10 mM HEPES (100 mM NaCl, 0.02% NaN_3, pH 7.4) buffer to a final peptide concentration of 100 μM. This solution was incubated at 37 °C with gentle shaking (77 rpm). The growth of the fibrils was followed by Thioflavin-T (THT) fluorescence measurements[17] until saturation was obtained. After the growth of the fibrils had reached its saturation point the solution was divided into aliquots for the disassembly studies. 0.15 M stock solutions were prepared by dissolving the organofluorine compounds or melatonin in DMSO; and this stock solution was added to the fibril samples in a 10:1 (small molecule:Aβ) molar ratio. After 30 s of vigorous vortexing the solutions were re-incubated at 37 °C with gentle shaking (77 rpm) and the decrease in fibril amount in each sample was periodically measured by THT fluorescence.[17] The fluorescence measurements were carried out using a Hitachi F-2500 fluorescence spectrophotometer. The incubated peptide solutions were briefly vortexed before each measurement, and then 3.5 μl aliquots of the suspended fibrils were withdrawn and added into freshly prepared 700 μl of 5 μM Thioflavin-T in 50 mM glycine–NaOH buffer (pH 8.5) and thoroughly mixed. The fluorescence spectra of these mixtures were measured at 430 nm (excitation) and 484 nm (emission) wavelengths, respectively. None of the tested compounds showed fluorescence intensity in this region. The Thioflavin-T fluorescence intensities (I_{THT}) were based on maximum fluorescence intensities in the 480–485 nm regions (emission spectra) after subtracting the background fluorescence of the Thioflavin-T alone. To calculate the relative fluorescence, the measured fluorescence intensities were normalized to the control sample containing the Aβ only (100%).

§ Aliquots of 2–5 μL from the control samples and disassembly assays were placed onto a freshly cleaved piece of mica. The samples were allowed to sit for 30–60 s. The excess peptide and buffer salts were carefully rinsed with de-ionized water and the specimen were air dried and subjected to analysis.

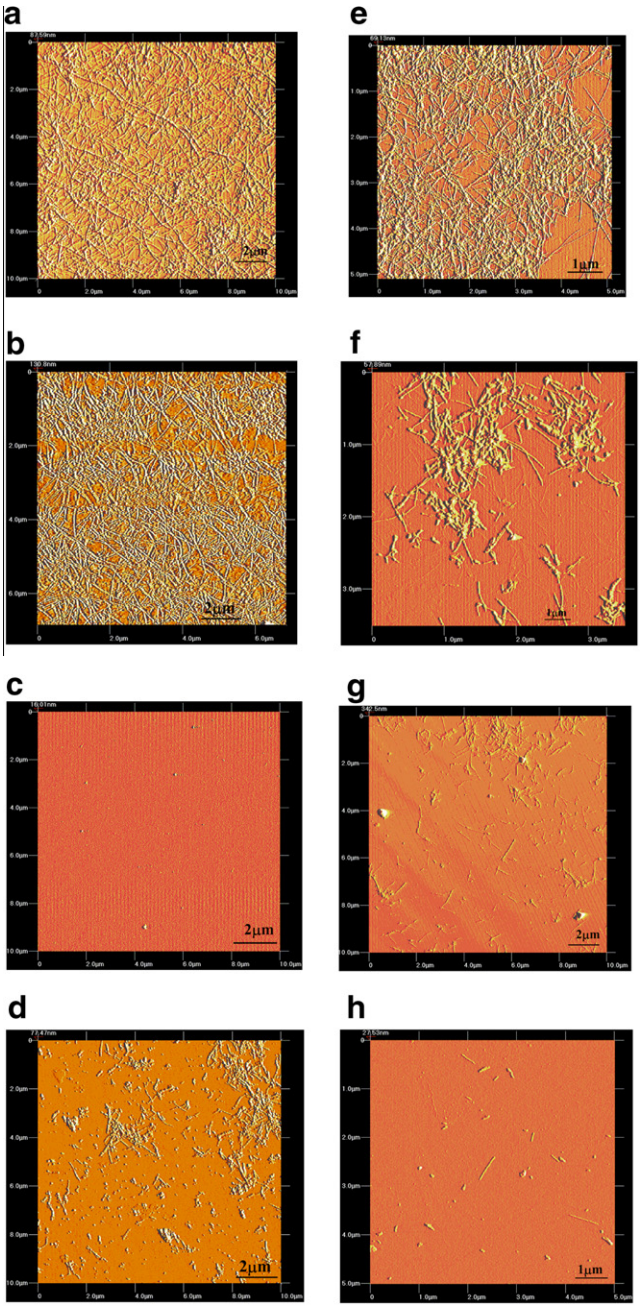

Figure 4. Atomic force microscopy images of $A\beta_{1-40}$ samples after the incubation of the preformed fibrils with the test compounds for the given time (a) C (control)-4 days, 10 μm^2 scan with *z*-axis of 87.59 nm, (b) **M**-4 days, 6 μm^2 scan with *z*-axis of 130.8 nm (c) **9**(*S*)-4 days, 10 μm^2 scan with *z*-axis of 16.01 nm, (d) **7**(*S*)-4 days, 10 μm^2 scan with *z*-axis of 77.40 nm, (e) **9**(*R*)-0 day, 5 μm^2 scan with *z*-axis height of 69.13 nm, (f) **9**(*R*)-1 day, 3 μm^2 scan with *z*-axis height of 57.89 nm, (g) **9**(*R*)-4 days, 10 μm^2 scan with *z*-axis of 342.5 nm, (h) **9**(*R*)-6 days, 5 μm^2 scan with *z*-axis height of 27.53 nm.

The AFM images correlate well with the fluorescence data. The image of the control shows well-developed fibrils as expected (Fig. 4a). There is no significant morphological change in the presence of melatonin either (Fig. 4b). However, samples incubated with the small organofluorine compounds, clearly showed disaggregated fibrils. The process of disaggregation could be observed unambiguously in the case of iodo-derivatives (**9**(*S*) Fig. 4c, and **9**(*R*) Fig. 4e–h) as well as **7**(*S*) (chloro-derivative, Fig. 4d). The efficacy order in which the molecules disassembled the fibrils, irrespective of the stereospecificity, is **9**, **8** and **7**. Figure 4e–h illustrates the disassembly process as a function of time. The images depict the gradual disassembly by **9**(*R*) from the starting point (0 day Fig. 4e) through day 1 (Fig. 4f) and day 4 (Fig. 4g) to day 6 (Fig. 4h).

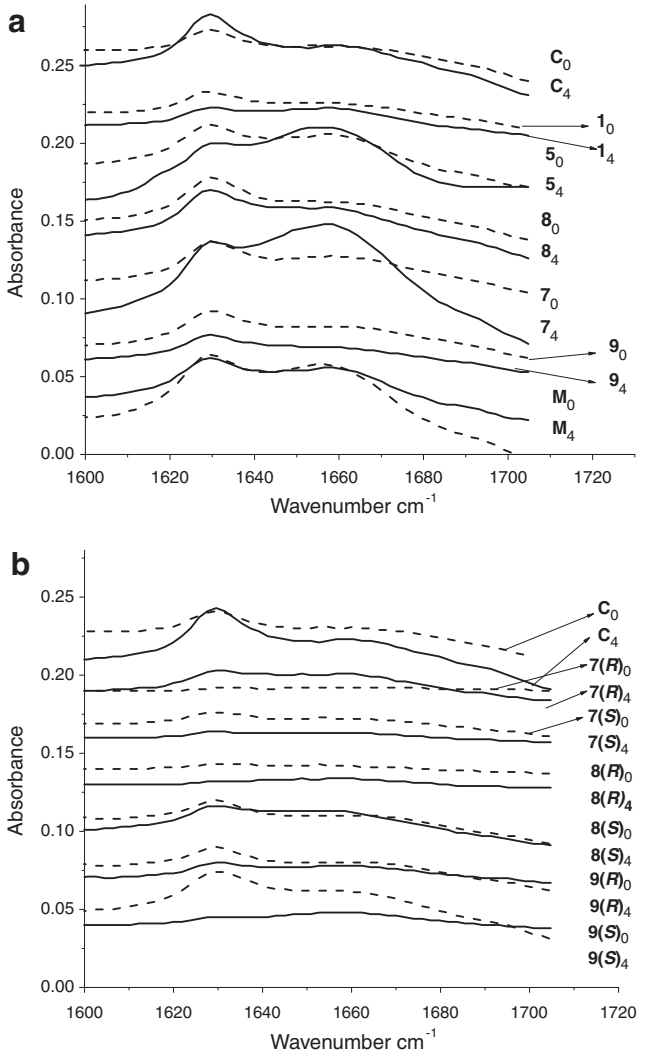

Figure 5. The absorbance FTIR spectra (showing the characteristic amide I region) obtained on the day 0 (dotted line) and on the day 4 (solid line) of the disassembly experiments in the presence of (a) selected racemic organofluorine compounds and melatonin (b) selected chiral organofluorine compounds. The label C indicates the control Aβ samples with no affector molecules added.

Structural changes due to the disassembly of fibrils were also followed by Fourier-transform infrared spectroscopy (FT-IR) focusing on the amide I (~1650 cm^{-1}) region of the absorption spectra (Fig. 5). The amide I vibrations are mainly attributed to the C=O stretches in the polypeptide backbone and most commonly used to perform secondary structure analysis.[19,**] Spectra obtained on day 0 of disassembly were dominated by an amide I vibration at 1630 cm^{-1} characteristic of β-sheet rich secondary structures (**C_0** and **C_4** in Fig. 5a and b).[19] The frequency of the dominating band is shifted from 1630 cm^{-1} to around 1645–1650 cm^{-1} indicating the formation of oligomers or other less ordered species. In agreement with the THT fluorescence and AFM results, the FT-IR data also indicated that the iodo-derivatives were the most efficient in disassembling the fibrils as observed by the shifts in the peak frequencies (**9_0**

** Aliquots of 20 μl from the incubated samples were withdrawn, lyophilized and mixed with 0.2 g solid KBr. The absorbance FT-IR spectra were recorded by a Nexus 870 FTIR spectrophotometer, *Omnic 7.1* was used for data collection and *Origins* was used for graphing.

and $\mathbf{9}_4$ in Fig. 5a). The appearance of a broad featureless band at around 1650 cm^{-1} was attributed to random coil structures.[18]

Other organofluorine derivatives studied were also able to disassemble the fibrils to some extent ($\mathbf{7}_0$ and $\mathbf{7}_4$, $\mathbf{8}_0$ and $\mathbf{8}_4$ in Fig. 5a), and this property was independent on the chirality (**7**(*R*), **7**(*S*), **8**(*R*), **8**(*S*), **9**(*R*), **9**(*S*) in Fig. 5b). Melatonin, which is a known inhibitor, was used as reference and it was observed that despite its good inhibiting potential, it was not able to disassemble the preformed fibrils ($\mathbf{M}_0$ and $\mathbf{M}_4$ in Fig. 5a).

The concordant data obtained by the various analytical methods clearly support the conclusion that 5′-halogen substituted 3,3,3-trifluoromethyl-2-hydroxyl-(indol-3-yl)-propionic acid esters tested above have a good potential for disassembling the mature fibrils, and this ability is irrespective of the stereospecificity associated with each compound. Since the same molecules were also good fibrillogenesis inhibitors,[13,14] these compounds can be classified as 'dual nature inhibitors'. This dual action makes them promising candidates for further drug development.

Acknowledgments

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