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Steroid−Quinoline Hybrids for Disruption and Reversion of Protein Aggregation Processes

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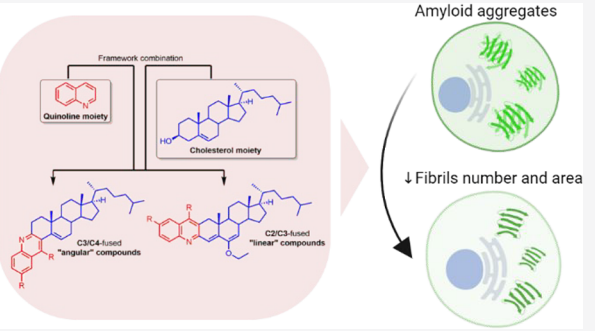
ABSTRACT: Reversing protein aggregation within cells may be an important tool to fight protein-misfolding disorders such as Alzheimer's, Parkinson's, and cardiovascular diseases. Here we report the design and synthesis of a family of steroid−quinoline hybrid compounds based on the framework combination approach. This set of hybrid compounds effectively inhibited Aβ1−42 self-aggregation *in vitro* by delaying the exponential growth phase and/or reducing the quantity of fibrils in the steady state. Their disaggregation efficacy was further demonstrated against preaggregated Aβ1−42 peptides in cellular assays upon their endocytosis by neuroblastoma cells, as they reverted both the number and the average area of fibrils back to basal levels. The antiaggregation effect of these hybrids was further tested and demonstrated in a cellular model of general protein aggregation expressing a protein aggregation fluorescent sensor. Together, our results show that the new cholesterol−quinoline hybrids possess wide and marked disaggregation capacities and are therefore promising templates for the development of new drugs to deal with conformational disorders.



KEYWORDS: *Steroid−quinoline hybrids, protein aggregation, amyloid-β (Aβ) peptide, protein misfolding diseases*

Protein aggregation is the process by which misfolded proteins adopt conformational changes that convert them from a physiologically soluble monomeric form into oligomeric and fibrillar forms, usually ones that are rich in stable β-sheet regions.[1,2] Many neurodegenerative diseases, such as Alzheimer's disease (AD), Huntington's disease, Parkinson's disease (PD), and prion diseases, as well as cardiovascular diseases (atherosclerosis, heart failure, and ischemic heart disease) are associated with protein aggregation. In some of these, smaller oligomeric forms of misfolded (amyloidogenic) proteins have been implicated as a causative agent.[3−5] Although this is not yet fully understood, over the last decades several efforts have been made to understand the molecular processes at the basis of such pathological aggregation by studying their formation and the complex network of chaperones, proteasome, and other regulatory molecules involved in their clearance.[6] These efforts have led to the development of several compounds, including natural products, peptides, and synthetic small molecules, to target many types of protein aggregation.[7,8] In recent years, steroids have emerged as alternative compounds to target not only amyloid-β (Aβ) aggregation but also other protein aggregation processes.[9−11] The seminal example is lanosterol, which because of its amphipathic nature binds to the hydrophobic sites of protein aggregates and exposes its hydrophilic part, causing lanosterol-bound proteins to be more soluble in water.[10,11] These features allow lanosterol to dissociate mutant cellular crystallin aggregates and to inhibit the self-assembly of Aβ entangling with peptides and interfering with the steric zipper interaction at the β-sheet−β-sheet interface.[9,11] In the last case, cholesterol was shown to be less efficient in the inhibition of Aβ aggregation because of its lower hydrophobicity compared with lanosterol.[9] Having that in mind, we focused our attention on the cholesterol molecule (which is readily available and much cheaper than lanosterol) and introduced chemical modifications on it to prepare hybrid cholesterol derivatives. Since quinolines have often been reported as good inhibitors of several types of protein aggregation processes, including Aβ aggregation,[12−18] we

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decided to fuse them to cholesterol, expecting to observe a synergic effect of the quinoline and steroid scaffolds (Figure 1).

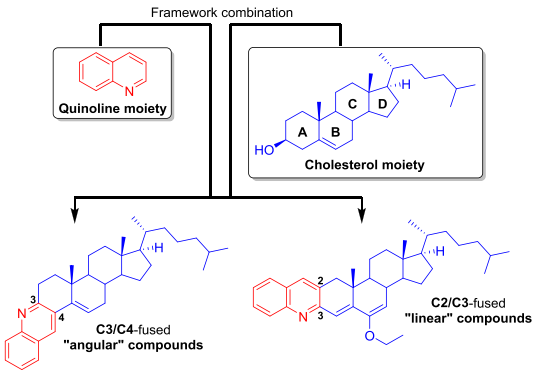


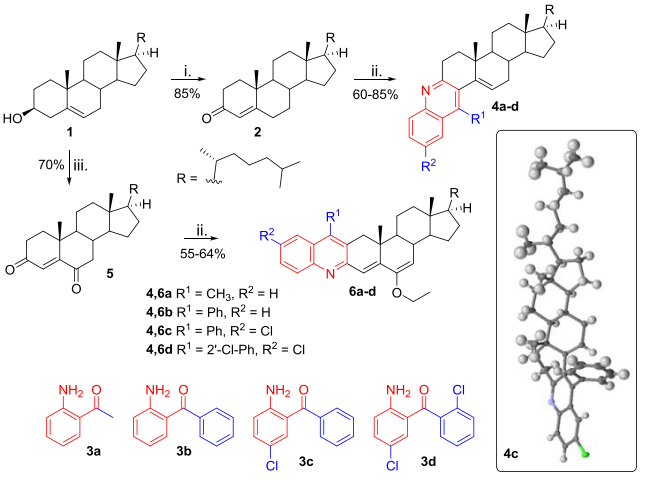
Figure 1. Framework combination to prepare "angular" and "linear" quinoline−steroid hybrids.

As we also introduced aromatic rings, the final hybrids were expected to be more hydrophobic than their cholesterol precursor (Figure 1). In addition, quinolines are also very interesting scaffolds for the development of fluorescent staining diagnostic tools for Aβ, tau, and PrP^{Sc} fibrils, as they are appealing binders of both amyloid and prion fibrils.[14,19−21] With this "framework combination" strategy, which was successfully applied previously to novel anticancer agents,[22] the hybrid compounds (Figure 1) will have aromatic/hydrophobic areas and hydrogen-bonding components, which are common structural features found in most Aβ inhibitors.[8]

The quinoline moiety was arranged within the hybrid compounds in distinct manners to afford "linear" and "angular" compounds, which are fused at the C2/C3 and C3/C4 sides of steroid A ring, respectively (Figure 1).

Compounds **4a−d** and **6a−d** were synthesized following a similar strategy, but the two series started with different oxysteroid precursors (Scheme 1). Compounds **4a−d** were prepared in two steps: (1) Oppenauer oxidation of the 3-OH of cholesterol (**1**) with aluminum isopropoxide [$Al(O^iPr)_3$] to give cholest-4-en-3-one (**2**) in 85% yield (Scheme 1(i))[23] and (2) microwave-assisted Friedlander annulation between **2** and 2′-aminoketones **3a−d** to afford steroid−quinoline hybrid "angular" compounds **4a−d** in 60−85% yield (Scheme 1(ii)).[24] The synthesis of the "linear" compounds **6a−d** followed a similar two-step synthetic approach, differing only in the use of the oxysteroid precursor cholest-4-en-3,6-dione (**5**), which was prepared by oxidation of **1** with pyridinium chlorochromate (PCC) (Scheme 1(iii)).[25] Dione **5** was used as the substrate for the Friedlander reaction with 2′-aminoketones **3a−d**, affording quinoline−cholesterol hybrids **6a−d** in 55−64% yield (Scheme 1(ii)).[24] The structures of the target compounds were fully elucidated using ^1H and ^{13}C NMR spectroscopy and high-resolution mass spectrometry for all compounds and single-crystal X-ray diffraction for compound **4c**, which confirmed the "angular" orientation of the quinoline moiety (see the Supporting Information (SI) for full details) and the retention of the configurations of all asymmetric carbons and the position of the B-ring C5═C6 double bond of the cholesterol moiety.

Scheme 1. Synthesis of Hybrid Compounds 4a−d and 6a−d[a] and (inset) Crystal Structure of 4c



[a]Reagents and conditions: (i) $Al(O^iPr)_3$, cyclohexanone, toluene, reflux, 12 h; (ii) **3a−d**, *p*-TsOH, EtOH, MW, 100 °C, 15 min; (iii) PCC, CH_2Cl_2, rt, 24 h.

With all of the desired compounds prepared, we first investigated their biocompatibility with human cells using the reversible resazurin (alias alamarBlue) metabolic colorimetric viability assay.[26,27] The cytotoxicities of compounds **4a−d** and **6a−d** were assessed in HeLa cells exposed for 24 and 48 h to increasing concentrations. All of the compounds were found to be nontoxic or showed low toxicity at 24 and/or 48 h of exposure at 0.1, 1, 10, 50, and 100 μM (see SI for full details).

The activities of compounds **4a−d** and **6a−d** on the target protein aggregates were then investigated. First, we investigated the effects of compounds **4a−d** and **6a−d** on Aβ1−42 peptide aggregation by a thioflavin-T (ThT) fluorescence assay using quercetin as the positive control (Figure S24).[28] According to the fluorescence intensity curves (Figure 2), the fibrillation process of Aβ1−42 peptides (control) followed a standard nucleated-growth mechanism, defined by a lag phase, a rapid exponential growth phase, and a final equilibrium steady state. Once compounds **4a−d** and **6a−d** were introduced to the monomer peptide solution, the final fluorescence intensity was reduced, indicating inhibition of the aggregation of Aβ1−42 peptides (Figure 2). The single exception was derivative **6d**, which showed no effect on the aggregation of Aβ1−42 peptides. Deserved highlight should be given to derivatives **4d** and **6a**, with which the final fluorescence intensity was greatly reduced, indicating a high degree of aggregation inhibition (Figure 2). On the other hand, for derivatives **4b**, **4c**, **6b**, and **6c**, the reduction of the final fluorescence intensity was mild, suggesting relatively weaker inhibition of peptide aggregation (Figure 2).

The arrangement of the quinoline moiety within the hybrid compounds **4b**, **4c**, **6b**, and **6c** seems to be irrelevant for the inhibition of peptide aggregation, with no relevant differences in their fibrillation processes (Figure S24). On the other hand, for compounds **4a**, **4d**, **6a**, and **6d**, the quinoline arrangement apparently had huge effect on the inhibition of peptide aggregation (Figure S24). The simplest linear compound **6a** demonstrated the highest inhibition effect, closely followed by the angular compound **4d** (Figure S24).

The disaggregation capacities of compounds **4a−d** and **6a−d** were then examined in SH-SY5Y neuronal-like cells incubated with preaggregated synthetic peptide Aβ1−

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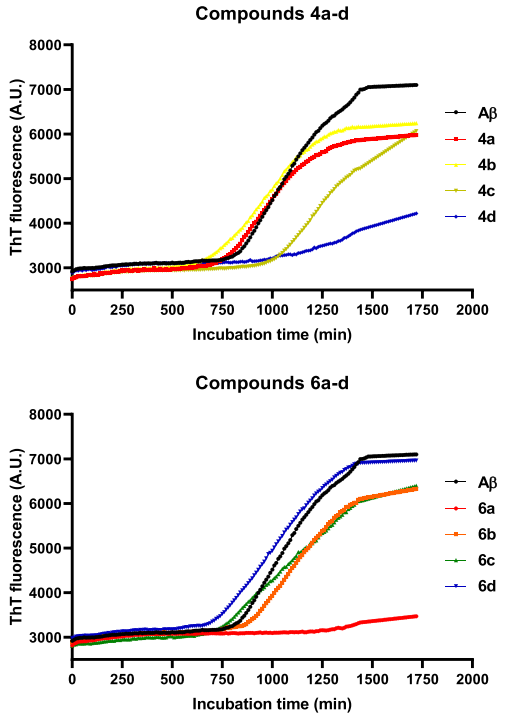

Figure 2. Influence of compounds **4a−d** and **6a−d** (20 μM) on fibrillation of Aβ1−42 peptides (10 μM) monitored by ThT fluorescence. Data are presented as the mean of three experiments (n = 3).

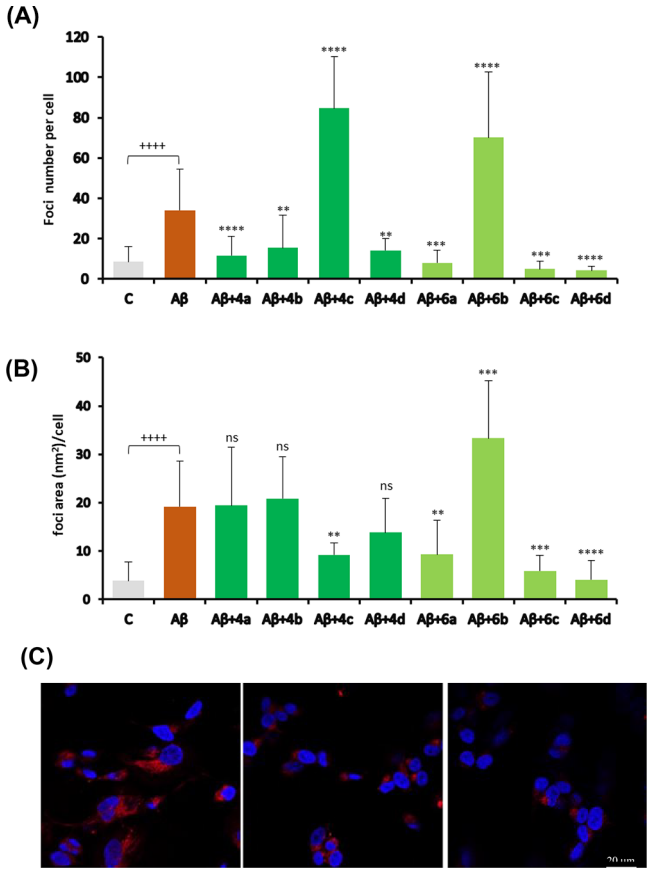

Figure 3. Evaluation of compounds' disaggregation effect in SH-SY5Y cells incubated with preaggregated Aβ1−42. (A) Numbers of protein aggregate foci per cell. (B) Average areas of protein aggregate foci per cell. Both control and Aβ-exposed cells were incubated in the presence of 0.5% EtOH (compound vehicle). Data are presented as mean ± SD (10−20 fields of view, n = 2, average of ∼370 cells analyzed per condition). Significance was determined using the unpaired Student's t test: (+) for comparison between positive (Aβ) and negative (C) control conditions and (*) for comparison between Aβ and Aβ + compounds. +/*, $p < 0.05$; ++/**, $p < 0.01$; +++/***, $p < 0.001$; ++++/****, $p < 0.0001$. (C) Micrographs of cells incubated with Aβ1−42 alone (left) or in combination with compound **4c** (center) or **6c** (right).

42.[29−31] After incubation with the aggregated Aβ1−42 peptides for 16 h, the cells were further incubated with each compound at 50 μM for an additional 12 h. Following incubation, the cells were fixed, stained with Proteostat protein aggregation assay,[32] and analyzed by confocal microscopy to determine whether there was a decrease in Aβ1−42 staining upon incubation with the tested compounds (Figure 3; see the SI for details). After incubation with the aggregated form of Aβ1−42, numerous red-fluorescent foci were detected throughout the cytoplasm (∼4-fold increase relative to the control cells that were in the presence of cellular medium only). An ∼5-fold increase in the average area of the foci was also observed, suggesting this as a suitable cellular model to screen the ability of compounds to disaggregate Aβ1−42 fibrils. Generally, the compounds were able to decrease the number of foci to basal levels, with the exceptions of derivatives **4c** and **6b**, which caused a large increase in the number of foci in comparison with Aβ1−42-incubated cells (Figure 3A). On the other hand, the average area of the foci was significantly reduced only in the presence of compounds **4c**, **6a**, **6c**, and **6d** (Figure 3B). The remaining compounds did not show any meaningful effect on the area of the foci, with the exception of **6b**, which caused an increase in the average area (Figure 3B). Figure 3C shows examples of confocal microscopy images for representative compounds **4c** and **6c**, in which one can observe a high number of red foci for compound **4c**, in contrast with compound **6c**.

Besides Aβ1−42 fibril formation, which is a hallmark of AD, there are various physiological- and pathological-relevant stresses that impact cell proteostasis and induce general protein aggregation. To mimic conditions of general protein aggregation and to test the ability of our synthetic compounds to reverse the random aggregation process, we used nilotinib (NTB), a clinically used anticancer drug that inhibits the tyrosine kinase protein Bcr-Abl[33,34] and induces proteostasis impairments, being employed to test protein aggregation sensors.[35,36] The disaggregation potential of the synthesized compounds was studied by incubating HeLa cells expressing the protein aggregation sensor HSP27:GFP[37] with NTB in the presence or absence of the tested compounds. Under protein-misfolding conditions, the HSP27:GFP sensor is relocalized to foci in cells, which can be detected by fluorescence microscopy.[37] If the tested compounds have the ability to disaggregate the misfolded proteins generated upon incubation with NTB, a decrease in the number and/or size of the HSP27:GFP foci will be detected (see the SI for details). To evaluate the disaggregation ability of compounds **4a−d** and **6a−d**, HSP27:GFP HeLa cells were incubated for 48 h with 5 μM NTB and coincubated with compounds **4a−d** and **6a−d** at 50 μM in the last 12 h of the incubation period. The assay outputs (number and area of protein aggregate foci per cell) are presented in Figure 4A,B. NTB increased the average number of cellular protein aggregates more than 5-fold, from

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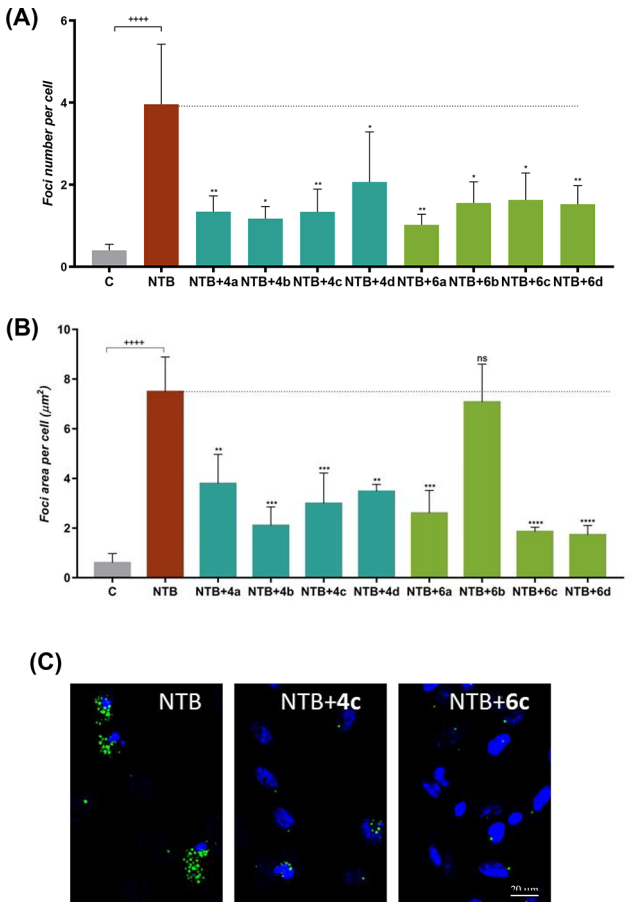

Figure 4. Analyses of the disaggregation capacities of compounds **4a–d** and **6a–d** under conditions of general protein aggregation. (A) Numbers and (B) average areas of green-fluorescing foci of protein aggregates per cell. Control and NTB-exposed cells were grown in the presence of 0.5% EtOH (compound vehicle). Data are presented as mean ± SD (n = 4–10, average of a total of ∼4700 cells analyzed per condition). Significance was determined using the unpaired Student's t test: (+) for comparison between positive (NTB) and negative (C) control conditions and (*) for comparison between NTB and NTB + compounds. +/*, $p < 0.05$; ++/**, $p < 0.01$; +++/***, $p < 0.001$; ++++/****, $p < 0.0001$. (C) Micrographs of representative cells of compounds **4c** and **6c**. Aggregates are in green (HSP27:GFP fusion protein) and cell nuclei in blue (DAPI).

0.71 ± 0.30 to 3.96 ± 1.39, as expected since NTB was seen to promote protein aggregation.[35] All of the synthesized compounds were able to significantly reduce the number of NTB-induced protein aggregates by ∼50–75%. The highest capacity was observed for **6a**, while **4d** demonstrated the lowest potential for decreasing the number of foci (Figure 4A). NTB also highly increased the average area of protein aggregates per cell (Figure 4B) by a factor of 12.5, from 0.6 ± 0.3 to 7.5 ± 1.3 μm^2. All of the synthesized compounds except **6b** were able to significantly reduce the area of NTB-induced protein aggregates, again by ∼50–75%. Compounds **6c** and **6d** showed the highest ability to reduce the area of the aggregates. Representative cells from NTB + **4c** and NTB + **6c** conditions are shown in Figure 4C.

In summary, eight new hybrid compounds designed by framework combination of quinoline and cholesterol scaffolds were synthesized and proved capable of inhibiting and reversing different types of protein aggregation. With this work, we found evidence that cholesterol could be used as template to create new compounds to deal with protein aggregation processes. The previously reported weaker suppressing effect of cholesterol on Aβ peptide aggregation was overcome by framework combination with a quinoline moiety. Our discovery is in line with previous conclusions demonstrating that compounds with higher hydrophobicity are better binders of Aβ oligomers. This hybridization strategy also proved capable of reversing general protein aggregation processes induced by nilotinib and might be interesting to deal with protein-misfolding diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.1c00604.

Spectra, biological assay data, and experimental procedures (PDF)

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#H.M.T.A. and R.N.d.S. contributed equally. The manuscript was written through contributions of all authors. All of the authors approved the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AD, Alzheimer's disease; PD, Parkinson's disease; PrP^{Sc}, scrapie prion protein; PCC, pyridinium chlorochromate; $Al(O^iPr)_3$, aluminum isopropoxide; *p*-TsOH, *p*-toluenesulfonic acid; ThT, thioflavin-T; NTB, nilotinib; HSP27:GFP, heat shock protein 27:green fluorescent protein

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