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Inhibition of amyloid-β aggregation by coumarin analogs can be manipulated by functionalization of the aromatic center

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ABSTRACT

Aggregation of the amyloid-β protein (Aβ) plays a pathogenic role in the progression of Alzheimer's disease, and small molecules that attenuate Aβ aggregation have been identified toward a therapeutic strategy that targets the disease's underlying cause. Compounds containing aromatic structures have been repeatedly reported as effective inhibitors of Aβ aggregation, but the functional groups that influence inhibition by these aromatic centers have been less frequently explored. The current study identifies analogs of naturally occurring coumarin as novel inhibitors of Aβ aggregation. Derivatization of the coumarin structure is shown to affect inhibitory capabilities and to influence the point at which an inhibitor intervenes within the nucleation dependent Aβ aggregation pathway. In particular, functional groups found within amyloid binding dyes, such as benzothiazole and triazole, can improve inhibition efficacy. Furthermore, inhibitor intervention at early or late stages within the amyloid aggregation pathway is shown to correlate with the ability of these functional groups to recognize and bind amyloid species that appear either early or late within the aggregation pathway. These results demonstrate that functionalization of small aromatic molecules with recognition elements can be used in the rational design of Aβ aggregation inhibitors to not only enhance inhibition but to also manipulate the inhibition mechanism.

1. Introduction

Current therapies for Alzheimer's disease (AD) target disease symptoms by increasing neurotransmission between surviving neurons to enhance memory and learning and thus improve quality of life. However, these therapies do not provide long-term benefits as they fail to treat the disease's underlying cause.[1,2] Leading theories suggest that amyloid plaques, which accumulate in the hippocampus and cerebral cortex, are the primary abnormality in AD. The principal component of these plaques, the amyloid-β protein (Aβ), is a proteolytic product of the transmembrane amyloid precursor protein (APP) and exhibits amphiphilic character as a result of its position within APP. Driven primarily by hydrophobic interactions, Aβ undergoes a nucleation dependent aggregation to produce ordered amyloid fibrils that deposit as plaques. This aggregation has been strongly implicated in disease progression.[1,3,4] Thus, preventing aggregation of Aβ represents a therapeutic strategy that directly targets AD pathogenesis.[1,5]

Numerous small molecules have been reported to interfere with the process of Aβ aggregation.[6–8] In particular, compounds containing aromatic centers are capable of preventing the assembly of monomeric Aβ into amyloid fibrils.[7,9,10] These compounds are hypothesized to interact with aromatic residues within the hydrophobic core of Aβ[11–15] and to inhibit aggregation by disrupting π-stacking of these aromatic residues, which is proposed to play a key role in amyloid aggregate formation and growth.[11] Less is known, however, about how the functionalization of aromatic centers can influence inhibitory capabilities. Among the limited reports of substituents that enhance inhibition are flexible hydrophobic moieties for functionalization of bisphenols;[12] hydroxy substitutions of the D-ring of apomorphorine;[13] polar, hydrogen bonding groups as substituents of aromatic centers within curcumin analogs;[14] the positioning of carboxylic acid groups and the optimal length of the linker between aromatic centers for anthranilic acid analogs;[15] and conformational flexibility of linkers that separate aromatic centers within derivatives of anthracycline, tetracycline, and carbazole.[16] Expanding the knowledge of functional groups that

Abbreviations: Aβ, amyloid-β protein; AChE, actylcholinesterase; AD, Alzheimer's disease; APP, amyloid precursor protein.

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improve inhibitory capabilities of aromatic centers will facilitate the rational design of effective Aβ aggregation inhibitors.

The current study employs a library of coumarin analogs, shown in Scheme 1, to both investigate novel aromatic inhibitors of Aβ aggregation and characterize substituents that enhance inhibitory capabilities. Coumarin (2*H*-chromen-2-one) is a naturally occurring aromatic compound found in cinnamon-flavored foods.[17] Coumarin derivatives exhibit good cell permeability, have been shown to be tolerated physiologically, and possess a broad range of pharmacological properties, including anti-coagulant, anti-tumor, and anti-inflammatory activities.[18] Recently, a derivative of coumarin was employed as a binding motif for the peripheral site of acetylcholinesterase (AChE) and successfully acted as a dual-function inhibitor for both AChE neurotransmitter breakdown and AChE-induced Aβ aggregation.[19] Due to the advantageous synthetic accessibility of coumarin, a library of coumarin analogs decorated with various functional motifs can be readily constructed.[20] Therefore, in the current study, coumarin is functionalized with small inorganic structures or nitrogen-containing ring structures homologous to those comprising amyloid binding dyes. The effect of coumarin analogs is monitored throughout the aggregation pathway to determine whether a compound intervenes prior to nucleation, indicative of interaction with protein monomers or small unordered aggregates, or post-nucleation, suggesting interaction with ordered aggregate structures. These studies identify coumarin analogs as novel inhibitors of Aβ aggregation. In addition, functionalization of coumarin with amyloid binding motifs is demonstrated to both enhance inhibition and influence the point at which a compound intervenes within the aggregation pathway, illustrating the potential that recognition elements have in the rational design of small molecule Aβ aggregation inhibitors.

2. Results

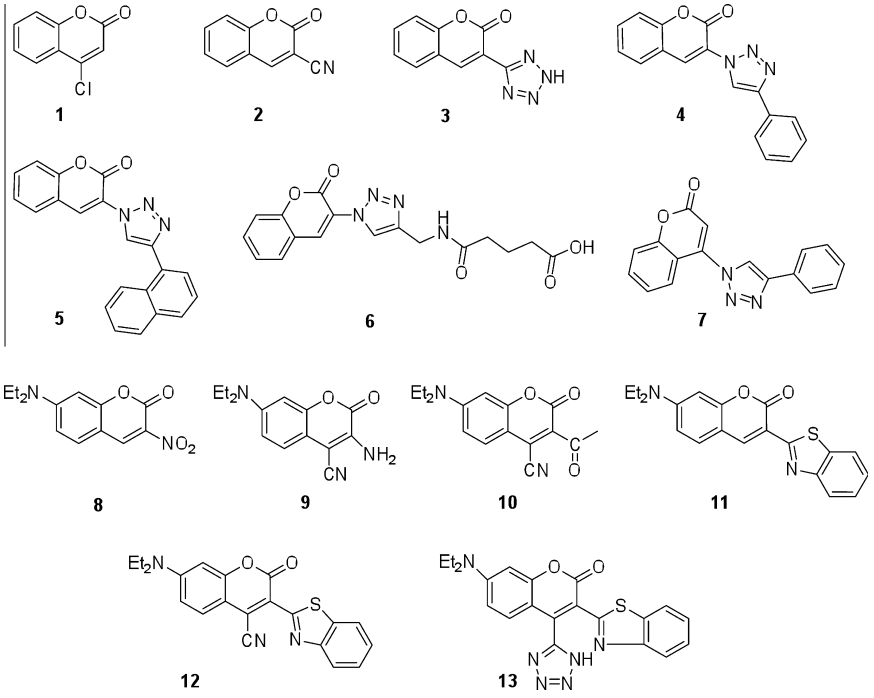
2.1. Synthesis of coumarin analogs

The structures of coumarin analogs tested in this study are shown in Scheme 1. Most of the syntheses followed reported protocols.[20–23] The formation of the triazole rings used the standard Cu(I)-catalyzed alkyne-azide cycloaddition reaction initiated from coumarin azides.[20,24,25] Cyanocoumarins were synthesized by direct cyanation with sodium cyanide at room temperature (Scheme 2), which could readily react with sodium azide to afford tetrazole analogs **3** and **13**, using Sharpless tetrazole synthesis.[26]

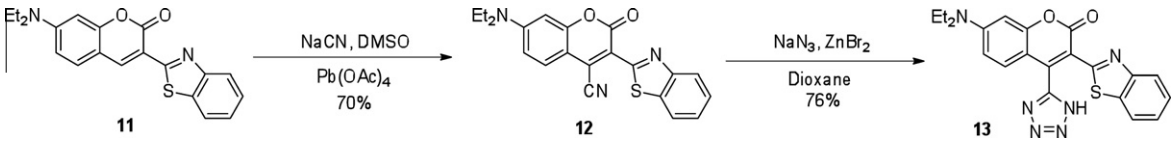
2.2. Inhibition of $A\beta_{1\text{-}40}$ monomer aggregation

In vitro studies have shown that Aβ fibrils formed using synthetic proteins are similar to fibrils isolated from amyloid plaques;[4] therefore, the synthetic form of the 40-residue Aβ protein, $A\beta_{1-40}$, was used to investigate the inhibitory capabilities of coumarin analogs. $A\beta_{1-40}$ is the predominant Aβ isoform within mature amyloid deposits[27] as well as the most abundant monomeric Aβ isoform in vivo.[3] Aggregation of $A\beta_{1-40}$ monomer was induced by continuous agitation and the presence of salt. The formation of β-sheet structured aggregates, monitored using thioflavin T fluorescence, exhibits an initial lag phase indicative of nucleation, subsequent aggregate growth, and a plateau reflective of fibrillar and soluble aggregates in equilibrium with monomer (Fig. 1). These kinetic stages are well established for both $A\beta_{1-40}$ and the longer 42-residue isoform of the protein, $A\beta_{1-42}$, with the kinetics for the two isoforms differing only in rate.[28] The slower kinetics exhibited by $A\beta_{1-40}$ make this isoform more suitable for studying early events within the Aβ aggregation pathway.

To assess inhibition at both early and late points within the Aβ aggregation pathway, changes in the lag and plateau phases of the aggregation curve were evaluated. An extension of the lag phase indicates the extent to which a compound can inhibit nucleation via interaction with Aβ monomer or small unordered aggregates. This measure of inhibition, which occurs early in the aggregation pathway, was determined as the ratio of the time at which thioflavin T fluorescence is first increased in the presence and absence of inhibitor, t_E and t_C, respectively (Fig. 1). A reduction in the equilibrium plateau indicates the extent to which a compound can reduce the quantity of aggregates containing β-sheet structure. If observed in the absence of a lag extension, this measure of inhibition



Scheme 1. Structures of coumarin analogs evaluated for their ability to inhibit Aβ aggregation.



Scheme 2. Synthesis of selected compounds.

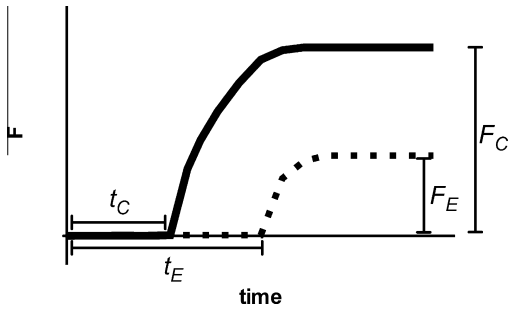


Figure 1. Assessment of inhibition of $A\beta_{1-40}$ monomer aggregation by coumarin analogs. Monomer aggregation assays were performed in the absence (solid line, control) and presence (dotted line, experimental) of the coumarin analog of interest, and aggregate formation was monitored via thioflavin T fluorescence (F) to evaluate each compound's ability to alter both the lag time to aggregate formation and the equilibrium plateau indicative of the quantity of aggregate formed. Extension of the lag time was evidenced as an increase in the experimental lag time, t_E, relative to the lag time observed for the control, t_C, and is evaluated as a ratio, t_E/t_C. Reduction of the equilibrium plateau was evidenced as a decrease in the experimental fluorescence plateau, F_E, relative to the fluorescence plateau observed for the control, F_C, and is evaluated as the percentage decrease from the control, $[(F_C - F_E)/F_C] \times 100\%$.

indicates intervention at late points during the aggregation pathway via interaction with ordered aggregates. Reductions in the quantity of β-sheet aggregates formed were determined as the percentage by which the plateau thioflavin T fluorescence in the presence of inhibitor, F_E, is reduced relative to the plateau observed in the absence of inhibitor, F_C (Fig. 1).

Some aromatic small molecules have been observed to disrupt the binding of thioflavin T to amyloid β-sheet structure and thus decrease thioflavin T fluorescence signals.[24,25] This disruption can render the thioflavin T method subject to false positives when it is used to identify inhibitors of Aβ aggregation. Other aromatic small molecules, however, do not impact this measurement technique.[29] To ensure that the coumarin analogs to be assessed as potential Aβ aggregation inhibitors (Scheme 1) fell within the latter category, the ability of each molecule to interfere with aggregate detection via thioflavin T fluorescence was evaluated. These measurements were acquired when coumarin based compounds were present at a fivefold molar excess over Aβ, the stoichiometric ratio present in samples used to monitor Aβ aggregation reactions. Thioflavin T detection of $A\beta_{1-40}$ fibrils formed in the absence of small molecules remained unchanged in the presence of each compound ($p > 0.05$) (data not shown). Additionally, fluorescence characterization of coumarin analogs was performed in the absence of $A\beta_{1-40}$ fibrils. Excitation and emission profiles confirmed that, at concentrations present in samples used to monitor Aβ aggregation, these compounds do not exhibit any inherent fluorescence that would compromise thioflavin T measurements (data not shown). Together, these results confirm that thioflavin T fluorescence can be used to accurately detect changes in Aβ aggregate formation that result from the presence of these potential small molecule inhibitors.

Representative results for $A\beta_{1-40}$ monomer aggregation in the presence of three compounds are shown in Figure 2, and the results for all synthesized coumarin analogs are summarized in Table 1 with respect to their effect upon both lag extension and equilibrium plateau reduction. Compound **1** had no inhibitory effect upon aggregation of $A\beta_{1-40}$ monomer (Fig. 2A). In fact, this compound slightly promoted aggregation as evidenced by a decrease in the lag time and an increase in the equilibrium plateau relative to the control. A similar absence of inhibitory activity was observed for only one other compound within the library, compound **6** (Table 1). In contrast, compound **5** exhibited strong inhibitory capabilities (Fig. 2B), doubling the lag time and reducing

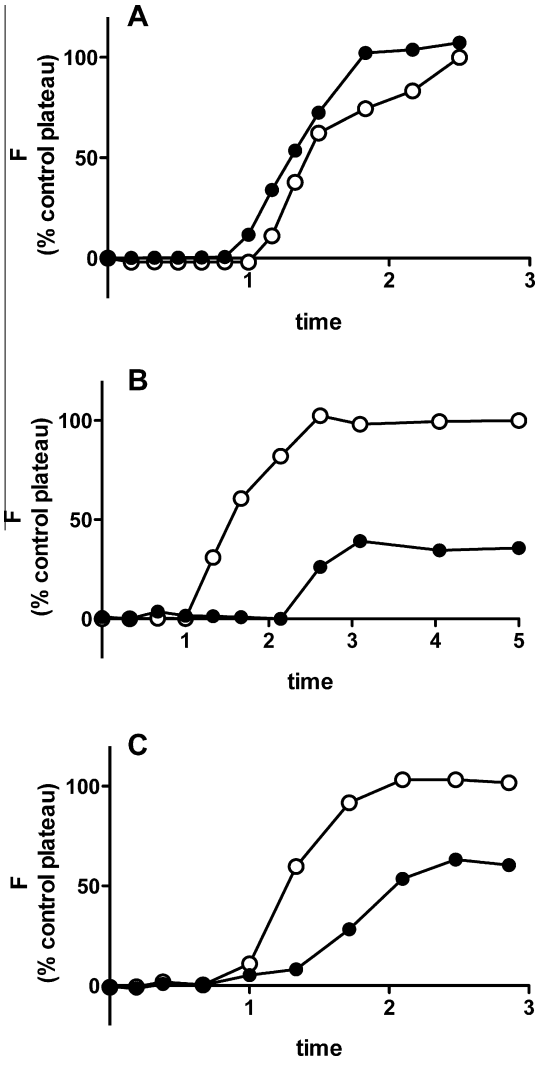


Figure 2. Effect of selected coumarin analogs on aggregation of $A\beta_{1-40}$ monomer. $A\beta_{1-40}$ monomer diluted to 20 μM in 40 mM Tris–HCl, pH 8.0, containing 150 mM NaCl and 2.5% (v/v) DMSO was incubated alone (control, ○) or in the presence of 100 μM compound **1** (panel A), compound **5** (panel B), or compound **12** (panel C) (●). Aggregate formation was induced by continuous agitation and monitored using thioflavin T fluorescence. Fluorescence values are plotted as a percentage of the equilibrium plateau fluorescence observed in the absence of compound, and time is plotted as a fraction of the lag time observed in the absence of compound. Results are representative of three independent experiments.

Table 1
Inhibition of $A\beta_{1-40}$ monomer aggregation by coumarin analogs[a,b]

Compound	Lag extension[c] (fold increase)	Plateau reduction [d] (% inhibition)
1	Promotion	Promotion
6	Promotion	Promotion
9	2.1 ± 0.1***	45 ± 5***
8	1.7 ± 0.2*	60 ± 5***
5	2.0 ± 0.1**	61 ± 4***
10	1.8 ± 0.2**	79 ± 3***
4	2.2 ± 0.3***	88 ± 1***
2	1.0 ± 0.0	22 ± 4**
12	1.1 ± 0.1	38 ± 4***
11	1.3 ± 0.1	64 ± 1***
7	1.4 ± 0.4	73 ± 2***
3	1.0 ± 0.2	87 ± 3***
13	1.0 ± 0.0	96 ± 1***

*p <0.05, **p <0.01, and ***p <0.001 compared to control.
[a] Monomer aggregation experiments were performed as in Figure 2.
[b] Parameters are expressed as mean ± SE, n = 3–5.
[c] Extension of the lag time to aggregate formation is expressed as a fold increase over the lag time observed for the control, as described in Figure 1.
[d] Reduction of the equilibrium plateau is expressed as a percentage of reduction in the equilibrium plateau observed for the control, as described in Figure 1.

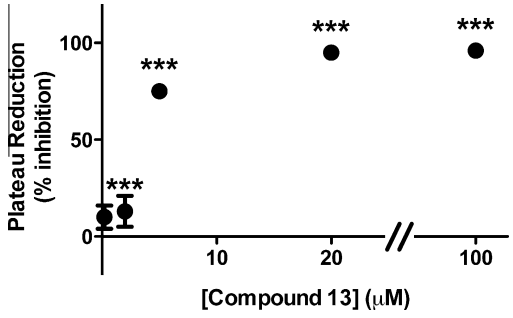


Figure 3. Effect of compound **13** on aggregation of $A\beta_{1-40}$ monomer. Aggregation of 20 μM $A\beta_{1-40}$ monomer was performed as in Figure 2 alone or in the presence of 0.2, 2, 5, 20, or 100 μM compound **13**. Results are expressed as a percentage of reduction in the equilibrium plateau fluorescence observed for the control, as described in Figure 1. Extension of the lag time was not observed at any concentration. Error bars represent SE, n = 3; some error bars lie within symbols. ***p <0.001 compared to control.

the equilibrium plateau by over 60%. Four other compounds also exhibited a significant effect upon both the lag time and the equilibrium plateau when compared to control aggregations performed in the absence of coumarin analog (Table 1). The impact of these compounds upon the initial lag phase was similar; however, the ability of these compounds to reduce the equilibrium plateau varied significantly. The compound exhibiting the weakest effect on this measure of inhibition, compound **9**, reduced the quantity of aggregates formed by 45 ± 5%, while the other compounds displaying this pattern of inhibition elicited greater reductions in the equilibrium plateau, ranging up to 88 ± 1%. Compound **12** also exhibited inhibitory capabilities; however, these capabilities were limited to a reduction in the equilibrium plateau, while the initial lag phase was not significantly altered (Fig. 2C). A similar pattern of inhibition was observed for five other compounds (Table 1), with the extent of reduction in the equilibrium plateau ranging from 22 ± 4% for compound **2** to a near complete inhibition of 96 ± 1% for compound **13**. In fact, compound **13** continued to reduce the equilibrium plateau as the concentration of inhibitor was reduced to 2 μM, where the compound was present at concentrations substoichiometric to Aβ (Fig. 3), illustrating the effectiveness of coumarin analogs as inhibitors of Aβ aggregation. Together, these results demonstrate how small molecule structures that contain an identical aromatic coumarin base but differ in their aromatic substitutions can exhibit diverse inhibitory capabilities with respect to delaying or reducing aggregation of Aβ.

3. Discussion

Inhibition of the assembly of Aβ monomer into aggregate structures has emerged as a therapeutic strategy for AD, and numerous small molecule inhibitors of Aβ aggregation have been identified.[6–8] In particular, compounds containing aromatic structures are reported to inhibit Aβ aggregation via disruption of π-stacking interactions between aromatic protein residues, but information about functional groups capable of enhancing inhibition by aromatic centers remains limited. Such information, on the other hand, will be influential in the rational design of therapeutics that target Aβ aggregation. In the current study, we identify analogs of the naturally occurring coumarin molecule as novel aromatic inhibitors of Aβ aggregation. By examining the effect of coumarin based compounds upon both early and late steps within the nucleation dependent Aβ aggregation pathway, we also provide evidence that functional groups containing amyloid binding motifs can serve as recognition elements to improve inhibition and define the point at which an inhibitor intervenes within the aggregation pathway.

Eleven of the coumarin analogs investigated exhibited inhibitory capabilities, ranging from a modest reduction of the equilibrium plateau (compound **2**) to nearly complete inhibition that extends to substoichiometric concentrations (compound **13**). These results demonstrate the strong potential of functionalized coumarin structures to attenuate Aβ aggregation. Coumarin derivatives have been shown to also intervene with other processes associated with symptomatic treatment and progression of AD, including slowing neurotransmitter breakdown via inhibition of AChE,[19,29,30] reducing the release of Aβ from APP via inhibition of β-secretase activity[30,31] or modulation of γ-secretase activity,[32] and serving as a free radical scavenger to protect cells against Aβ-induced toxicity.[33] The additional action of coumarin analogs as Aβ aggregation inhibitors further establishes these structures as potential multi-target AD therapeutics. In fact, the observed ability of orally administered coumarin analogs to attenuate Aβ-induced memory impairment in mice[34] could result from a combination of these actions.

A pronounced effect upon inhibition of Aβ aggregation was observed when coumarin was functionalized with nitrogen-containing ring structures benzothiazole and triazole, which are found within amyloid-binding dyes. Coumarin analogs containing benzothiazole reduced the extent of aggregate formation without significantly altering the lag time to aggregate appearance. In contrast, coumarin analogs functionalized with triazole both extended the lag time and reduced the amount of amyloid aggregates formed. Benzothiazole comprises the backbone of the amyloid binding dye thioflavin T as well as derivatives of this structure that have been synthesized as probes for in vivo imaging of fibrillar deposits.[6] Thioflavin T derivatives have been shown to exhibit a high binding affinity and a high specificity for binding aggregated Aβ deposits isolated from AD brain.[35,36] While some compounds that contain benzothiazole have been reported to inhibit Aβ aggregation,[37] other benzothiazole containing compounds[38] are unable to halt Aβ fibril formation. Triazole structures have been incorporated into fluorescent probes that bind amyloid plaques within AD brain sections[39] and Aβ aggregates prepared in vitro;[40] however, these compounds have not been investigated for their ability to inhibit Aβ aggregation. Triazole structures have been incorporated within Aβ aggregation inhibitors but only as a linker between functional groups of multifunctional molecules and not as an aggregation inhibitor moiety.[41] Thus, benzothiazole and triazole

may not impart upon coumarin based compounds the ability to disrupt aggregate formation and growth. Instead, benzothiazole and triazole groups may promote binding of coumarin analogs to Aβ and position other structural elements within the compound for disruption of aggregation. A role for interaction between the coumarin structure and Aβ in the disruption of aggregation, rather than simple steric hindrance, is indicated by reports that benzothiazole coupled to a different bulky aromatic group failed to inhibit Aβ aggregation.[38]

Cooperation between recognition and disruption elements has been demonstrated for peptide inhibitors of Aβ aggregation.[42] Extension of this premise to the small molecule inhibitors investigated in the current study is supported by the observed correlation between the binding capabilities of amyloid dyes and the inhibitory capabilities of the functionalized compounds. Benzothiazole based amyloid binding dyes recognize the β-sheet structure of amyloid aggregates but do not bind monomeric protein or unordered oligomers.[43,44] In contrast, incorporation of a triazole ring within boron-dipyrromethene dyes confers the ability to bind unordered Aβ oligomers as well as β-sheet aggregates.[40] Hence, triazole should facilitate inhibition of both early and late steps within the aggregation pathway, while benzothiazole should render compounds capable of interacting only with already formed aggregates and thus able to intervene solely at later stages within the aggregation pathway. Indeed, the observed data reflects these trends. Replacement of nitrile within compound **2** with a triazole functionalized by either a phenyl ring (compound **4**) or fused phenyl rings (compound **5**) both imparts the ability to extend the lag time to aggregate formation and enhances reduction of the equilibrium plateau. In contrast, benzothiazole replacement of nitro, amine, or carbonyl, within compounds **8**, **9**, and **10**, respectively, yields compounds **11** and **12** that are no longer able to extend the lag time but do reduce the equilibrium plateau. These results suggest that functionalization of coumarin with benzothiazole and triazole may lead to inhibitor recognition of Aβ structures that appear at different stages within the aggregation pathway.

The position at which a recognition element is located also influences the inhibitory capabilities of a compound. Location of the triazole group at position 3 of the coumarin base structure in compound **4** results in a compound that doubles the lag time and reduces the extent of aggregation by almost 90%. When this same triazole group is located instead at position 4 of the coumarin base structure (compound **7**), the resulting compound continues to significantly decrease the quantity of amyloid aggregates formed but has only a modest effect upon the lag time to aggregate formation. Thus, placement of this group at the end of the molecule, rather than rotating the group toward the interior of the structure may influence the manner in which the compound interacts with unordered aggregates. In addition, the triazole ring functionalized with a single phenyl group (compound **4**) exhibited a significantly stronger ability (p <0.001) to reduce the amount of amyloid material formed than did the triazole ring functionalized with fused phenyl rings (compound **5**). In contrast, when the triazole ring was functionalized with a long hydrophilic chain incorporating a negative charge (compound **6**), inhibitory capabilities were attenuated. Together, these results suggest that hydrophobicity, the absence of negative charge, or both are important in either facilitating binding to Aβ or disrupting Aβ aggregate formation and growth. Alternatively, the phenyl structure itself may play an important role, as multiple aromatic centers have been demonstrated to enhance inhibitory capabilities of other small molecules.[16]

Coumarin functionalization with another nitrogen-containing ring structure, tetrazole, also reduced the extent of aggregation without prolonging the lag time to aggregate formation, indicating that, like benzothiazole, tetrazole functionalized compounds work by binding already formed aggregates to disrupt growth. This inhibition was observed whether coumarin was functionalized with tetrazole alone, as in compound **3**, or in combination with other functional groups, as in compound **13**. Furthermore, tetrazole replacement of nitrile in compound **2** to give compound **3** and in compound **12** to give compound **13** led to a significant increase (p <0.001) in reduction of the equilibrium plateau, demonstrating the effectiveness of tetrazole functionalization for inhibitor performance. Tetrazole containing compounds have not yet been reported as inhibitors of Aβ aggregation, and these results demonstrate their potential for positively influencing inhibition. Furthermore, the coumarin analog substituted with both benzothiazole and tetrazole (compound **13**) exhibited the strongest ability among the investigated compounds to reduce the quantity of aggregates formed, illustrating that effective substituents can be combined to yield a compound with superior inhibitory activity. In fact, this compound continued to exhibit inhibitory capabilities down to a substoichiometric ratio of 1:10. The benzothiazole and tetrazole structures may recognize different binding sites within Aβ aggregates. Similar differences in ligand binding by Aβ aggregates have also been observed for amyloid dyes with distinct structures.[45,46] As a result, the enhanced inhibitory capabilities could result from the ability of this compound to disrupt multiple sites of aggregate growth.

4. Conclusions

The current study identifies coumarin analogs as novel inhibitors of Aβ aggregation, adding to the body of aromatic structures capable of modulating Aβ aggregate assembly. Unlike other aromatic inhibitors, however, coumarin analogs exhibit a number of additional AD-associated pharmacological effects and thus possess the potential to serve as multi-target AD therapeutics. The effects that functional substituents of the coumarin base have upon inhibitory capabilities demonstrate that amyloid binding motifs homologous to those found in amyloid binding dyes can enhance inhibitory capabilities. Furthermore, correlation between appearance of the type of Aβ aggregates that an amyloid binding motif recognizes and the point of inhibitor intervention within the multi-step aggregation pathway implicates a role for the recognition capabilities of these structural elements. Together, these results suggest that structures which function as recognition elements for small molecule inhibitors can be manipulated to both improve inhibition and control an inhibitor's mechanism of action. Moreover, these structural elements can be combined to further enhance inhibitory capabilities. As a result, understanding the effect that recognition elements have upon inhibition of both aggregate formation and growth has the potential to advance the rational design of Aβ aggregation inhibitors.

5. Experimental

5.1. Materials and general methods

Unless otherwise noted, all chemicals and solvents were obtained from commercial suppliers and used without purification. ^{1}H NMR spectra were recorded on a Bruker 300 NMR spectrometer and ^{13}C NMR spectra were recorded either on a Bruker 300 NMR or a Bruker 400 NMR spectrometer. $A\beta_{1-40}$ peptide was purchased from Anaspec, Inc. (San Jose, CA). Thioflavin T was obtained from Sigma (St. Louis, MO). Bovine serum albumin and DMSO were obtained from EMD Biosciences (San Diego, CA).

5.2. Synthesis of functionalized coumarin library

Compounds **1**,[21] **2**,[22] **4**-**7**,[20] and **8**–**11**,[23] (Scheme 1) were synthesized following the literature procedures, respectively.

5.2.1. 3-(2*H*-Tetrazol-5-yl)-chromen-2-one (compound 3)

In a 5 mL round bottomed flask was taken 3-cyanocoumarin (compound **2**, 100 mg, 0.58 mmol) followed by the addition of zinc bromide (152 mg, 0.58 mmol) and sodium azide (75 mg, 1.16 mmol). The resulting solid mixture was then heated to 100-150 °C for 24 h and then 150–200 °C for another 12 h. Upon cooling, the solid mixture was added with water (2 mL) and concd HCl (0.5 mL); the resulting suspension was stirred at room temperature for 30 min, filtered, washed with water and dried under vacuum to yield the product as a white solid; 60 mg (49%). IR (KBr): 1697 (vs), 1620 (s), 1573 (s), 1450 (m), 1412 (m); ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.80 (s, 1H), 7.52-7.89 (m, 2H), 7.45–7.49 (m, 2H).

5.2.2. 3-Benzothiazol-2-yl-7-diethylamino-2-oxo-2*H*-chromene-4-carbonitrile (compound 12)

The compound 3-benzothiazol-2-yl-7-diethylamino-chromen-2-one (compound **11**, 1 g, 2.86 mmol) was dissolved in 10 mL of DMSO and the resulting solution was stirred at room temperature for 10 min. Sodium cyanide (0.29 mg, 5.7 mmol) was then added before the reaction mixture was stirred at room temperature for 2 h. The dark brown colored solution was then added with lead tetraacetate (1.27 g, 2.86 mmol) at room temperature and stirred for 1 h (Scheme 2). The resulting solution was poured drop-wise into 200 g of ice and a pinch of sodium chloride was added and stirred for 1 h at room temperature. The precipitation was collected, washed with 50 mL water and dried under vacuum to yield a pink solid as the pure product. The crude product containing a trace amount of the starting material was purified by treatment under reflux for overnight using 20 mL of a solvent mixture containing 20% ethyl acetate and 80% hexane to yield 750 mg of pure product (yield: 70%). mp 237–239 °C; IR (KBr): 2221 (m), 1712 (m), 1620 (m), 1558 (m), 1504 (s), 1420 (m); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.20 (d, J = 7.95 Hz, 1H), 7.87–7.98 (m, 2H), 7.34–7.55 (m, 2H), 6.78 (d, J = 6.78 Hz, 1H), 6.55 (s, 1H), 3.48 (t, J = 6.94 Hz, 4H), 1.27 (t, J = 7.02 Hz, 6H); HRMS calcd for $C_{21}H_{17}N_3O_2S$: 375.1041, found: 375.1035.

5.2.3. 3-Benzothiazol-2-yl-7-diethylamino-4-(1*H*-tetrazol-5-yl)-chromen-2-one (compound 13)

Compound **12** (0.1 g, 0.27 mmol) was suspended in 5 mL of a mixed solvent containing 80% 1,4-dioxane and 20% water followed by the addition of zinc bromide (72 mg, 0.32 mmol) and sodium azide (21 mg, 0.32 mmol) (Scheme 2). The resulting mixture was heated to reflux using an oil bath for a period of 48 h. Upon cooling to room temperature, the solid mixture was added to 10 mL of water and the precipitated solid was filtered and washed with 10 mL of water and dried under vacuum. The crude product mixture was then dissolved in 5 mL of 1 M NaOH solution. The yellow filtrate was collected and mixed with 10 mL of water. The aqueous mixture was then extracted with 10 mL diethyl ether and gently shaken using a separating funnel to remove traces of unreacted cyanocoumarin starting material. The aqueous layer was acidified using 1:1 concd HCl to pH 4, and the mixture was gently stirred at 50 °C for 1 h to yield a yellowish brown suspension, which was then filtered, washed with 10 mL of water and dried under vacuum to yield the product as a brownish yellow solid; 85 mg (yield: 76%). mp >250 °C; IR (KBr): 1705 (m), 1621 (s), 1649 (s), 1573 (w), 1358 (m), 1265 (m); ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.31–7.91 (m, 5H), 6.57 (m, 2H), 3.44 (q, J = 7.64 Hz, 4H), 1.21 (t, J = 6.91 Hz, 6H); HRMS calcd for $C_{21}H_{18}N_6O_2S$: 419.1309, found: 419.1290.

5.3. Preparation of coumarin analogs for experimentation

Coumarin analogs were dissolved at 4 mM in DMSO and stored at −20 °C. Immediately prior to experimentation, stock solutions were diluted into final reactions for a concentration of 100 μM and 2.5% (v/v) DMSO. For dose-dependent experiments, stock solutions were diluted in DMSO such that subsequent dilution into final reactions resulted in varying concentrations of coumarin analog in the presence of a constant 2.5% (v/v) DMSO. DMSO (2.5% v/v) was added to all control reactions run in parallel.

5.4. Purification of $A\beta_{1-40}$ monomer

Lyophilized $A\beta_{1-40}$ peptide, stored desiccated at −20 °C, was prepared for experimentation as described previously.[47] Briefly, peptide was solubilized in 50 mM NaOH at a concentration of 2 mg/mL. To remove any pre-existing aggregates, solubilized preparations were purified via size exclusion chromatography on a Superdex 75 HR10/300 column (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) equilibrated in 40 mM Tris–HCl, pH 8.0, and pretreated with 2 mg/mL bovine serum albumin to reduce nonspecific interaction between the peptide and the column matrix. Monomer concentrations were calculated using UV absorbance (276 nm) and an extinction coefficient of 1450 L/mol cm.[28] Monomer was stored at 4 °C no longer than 4 days prior to use in experiments.

5.5. $A\beta_{1-40}$ monomer aggregation assay

The aggregation of $A\beta_{1-40}$ monomer was assessed as described previously.[29] Briefly, 20 μM $A\beta_{1-40}$ monomer was combined with 0 μM (control) or 0.2–100 μM coumarin analog in the presence of 40 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 2.5% (v/v) DMSO. Initial experimentation utilized a fivefold molar excess of coumarin analog over $A\beta_{1-40}$ monomer to give a robust effect for both weak and strong inhibitors and facilitate comparisons of inhibitory capabilities among coumarin analogs. Reactions were incubated at 25 °C with continuous agitation (vortex, 500 rpm). To monitor amyloid aggregate formation, aliquots were periodically diluted eightfold into 10 μM thioflavin T and evaluated for fluorescence using an LS-45 luminescence spectrometer (Perkin–Elmer, Waltham, MA) (excitation = 450 nm, emission = 470–500 nm). Fluorescence values (F) were determined by integrating the area under the emission curve with baseline (thioflavin T) subtraction. Extension of the lag time to aggregate formation induced by each compound was calculated as a fold change in the lag time to fluorescence increase relative to that observed in the absence of inhibitor; a value of 1 represents no difference from the control. Decreases in the total aggregate formation induced by each compound were calculated as a percentage of reduction in the plateau fluorescence value relative to that observed in the absence of inhibitor; a value of 0% represents no difference from the control. Three to five independent experiments were performed for each aromatic compound, and results are reported as the mean ± standard error.

5.6. Preparation of $A\beta_{1-40}$ fibrils

$A\beta_{1-40}$ fibrils were prepared for experimentation as described previously.[47] Briefly, 50–100 μM $A\beta_{1-40}$ monomer in 40 mM Tris–HCl, pH 8.0, was agitated (vortex, 800 rpm) in the presence of 250 mM NaCl at 25 °C for over 24 h. Fibrils, defined as pelletable aggregates, were separated from monomeric protein and soluble aggregates via centrifugation (14,000×g, 15 min). Fibrils were resuspended in 40 mM Tris–HCl, pH 8.0, and the fibril concentration was calculated from the fraction of pelleted protein. Fibrils were stored at 4 °C for no longer than 7 days prior to use in experiments.

5.7. Detection of $A\beta_{1-40}$ aggregates in the presence of coumarin analogs

To ensure that thioflavin T detection of aggregated $A\beta_{1-40}$ was unaffected by the presence of compounds within the library,

detection of pre-formed $A\beta_{1-40}$ fibrils was carried out in the presence of each synthesized compound. $A\beta_{1-40}$ fibrils were diluted to a concentration of 2.5 μM in 40 mM Tris–HCl, pH 8.0, containing 10 μM thioflavin T and combined with 0 μM (control) or 12.5 μM compound and 2.5% (v/v) DMSO. These concentrations reflect the final concentrations within diluted samples used to monitor $A\beta_{1-40}$ monomer aggregation. Solutions were incubated at 25 °C for 15 min to ensure binding, and thioflavin T fluorescence was evaluated as described above.

5.8. Statistical analysis

Statistical analysis was performed using Prism 5 software (GraphPad Software Inc., San Diego, CA). The effect of compound structure upon both lag extension and equilibrium plateau reduction as well as thioflavin T detection of pre-formed $A\beta_{1-40}$ aggregates was assessed using a one-way analysis of variance. Dunnett's test was used to assess differences in lag extension, equilibrium plateau reduction, and aggregate detection between experimental groups and controls. Tukey's test was used to assess differences in lag extension and equilibrium plateau reduction among experimental groups. $p < 0.05$ was considered significant.

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