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Heterodivalent Linked Macrocyclic β-Sheets with Enhanced Activity against Aβ Aggregation: Two Sites Are Better Than One

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Supporting Information

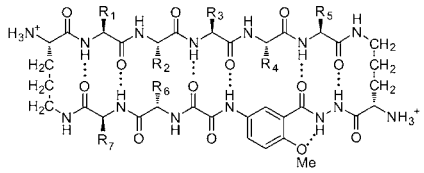
ABSTRACT: This paper reports a series of heterodivalent linked macrocyclic β-sheets **6** that are not only far more active against amyloid-β (Aβ) aggregation than their monovalent components **1a** and **1b** but also are dramatically more active than their homodivalent counterparts **4** and **5**. The macrocyclic β-sheet components **1a** and **1b** comprise pentapeptides derived from the N- and C-terminal regions of Aβ and molecular template and turn units that enforce a β-sheet structure and block aggregation. Thioflavin T fluorescence assays show that heterodivalent linked macrocyclic β-sheets **6** delay $Aβ_{1–40}$ aggregation 6−8-fold at equimolar concentrations and substantially delay aggregation at substoichiometric concentrations, while homodivalent linked macrocyclic β-sheets **4** and **5** and monovalent macrocyclic β-sheets **1a** and **1b** only exhibit more modest effects at equimolar or greater concentrations. A model to explain these observations is proposed, in which the inhibitors bind to and stabilize the early β-structured Aβ oligomers and thus delay aggregation. In this model, heterodivalent linked macrocyclic β-sheets **6** bind to the β-structured oligomers more strongly, because N-terminal-derived component **1a** can bind to the N-terminal-based core of the β-structured oligomers, while the C-terminal-derived component **1b** can achieve additional interactions with the C-terminal region of Aβ. The enhanced activity of the heterodivalent compounds suggests that polyvalent inhibitors that can target multiple regions of amyloidogenic peptides and proteins are better than those that only target a single region.

INTRODUCTION

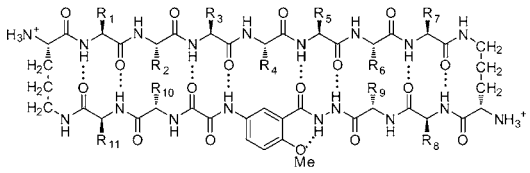
Amyloid-β (Aβ) fibrils associated with Alzheimer's disease contain layered β-sheet structures involving β-strands from both the N- and C-terminal regions of Aβ peptides.[1] NMR-based structural models of Aβ fibrils show that Aβ peptides self-assemble into parallel β-sheets that fold into U-shaped superstructures (Figure 1).[2] The two parallel β-sheets of the U-shaped superstructure are layered in an antiparallel fashion. Similar fibril structures also occur in human islet amyloid polypeptide associated with type II diabetes and likely occur more widely in amyloids.[3]

Macrocyclic β-sheets containing turn and template units provide useful chemical tools with which to understand and control amyloid aggregation.[4] Our laboratory has introduced 42- and 54-membered ring macrocycles **1** and **2** (Chart 1) that can fold into β-sheet structures and display preorganized β-strands. Macrocycle **1** incorporates a pentapeptide β-strand into the upper strand, while macrocycle **2** incorporates a heptapeptide β-strand. When these macrocycles display amyloidogenic β-strands, they are able to inhibit or suppress amyloid aggregation through β-sheet interactions. We have demonstrated that macrocycles **1** containing pentapeptide VQIVY can inhibit aggregation of the τ-derived peptide Ac-VQIVYK-NH_2 (AcPHF6) associated with Alzheimer's disease[5] and that macrocycles **2** containing amyloidogenic heptapeptide sequences can inhibit aggregation of Aβ, $β_2$-microglobulin and α-synuclein and can detoxify Aβ aggregates.[4c,d] We have also demonstrated that the activity of macrocyclic β-sheets against Aβ aggregation can be dramatically enhanced through expansion from macrocycle **1** to **2**.[4d]

Chart 1



macrocycle **1**



macrocycle **2**

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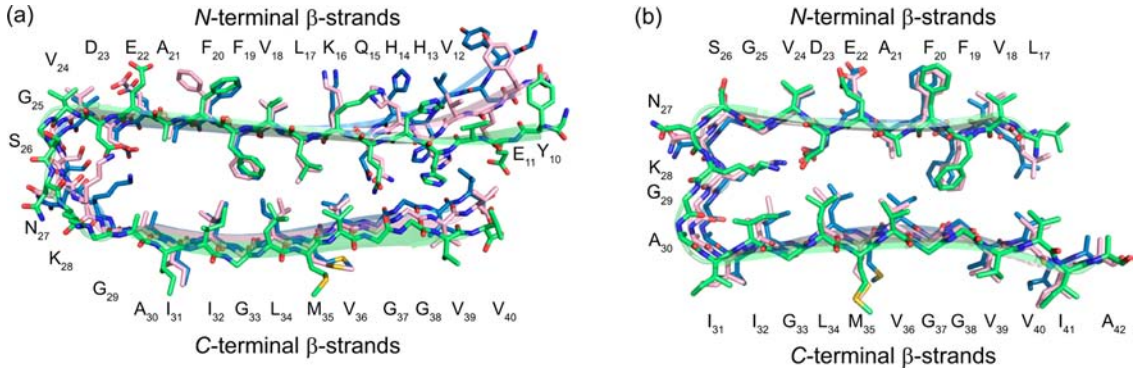
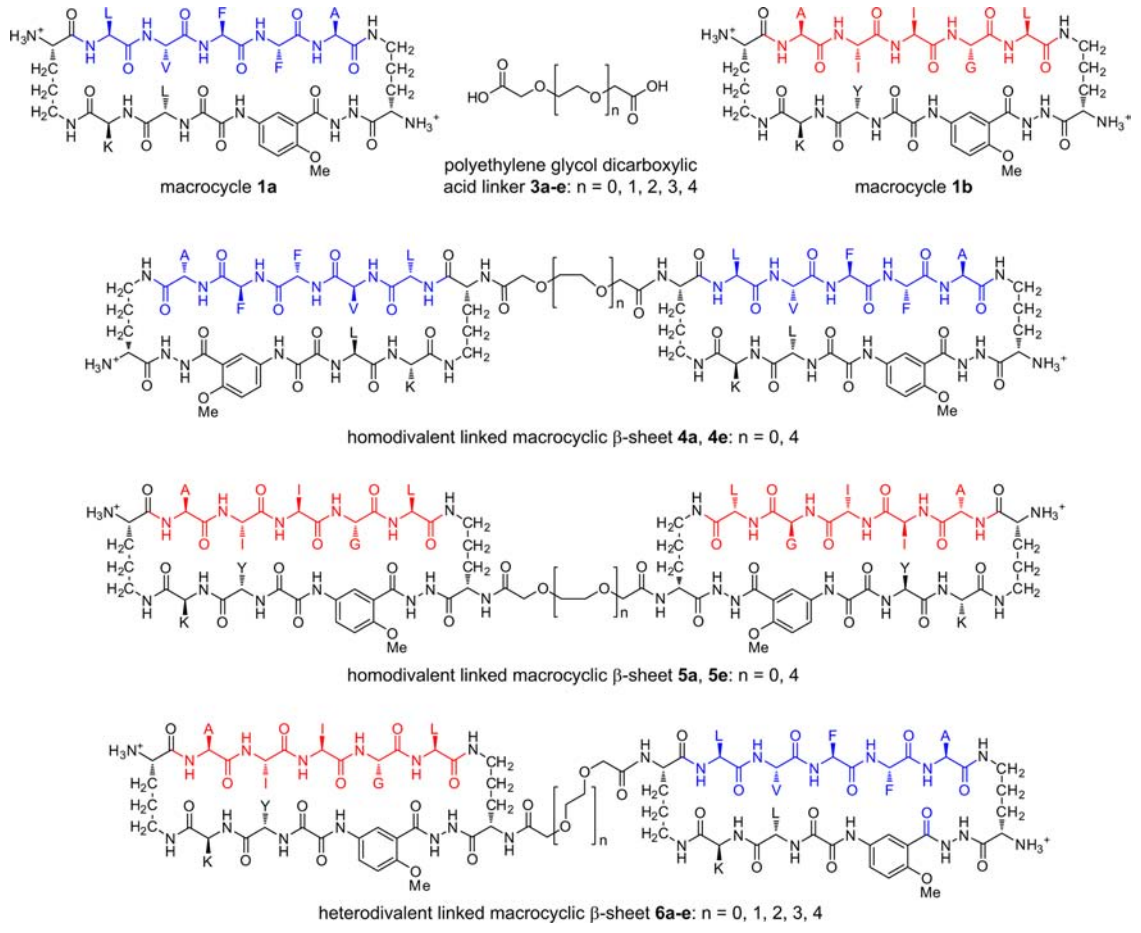


Figure 1. NMR-based structural models of Aβ fibrils. Models of (a) Aβ_{1-40} and (b) Aβ_{1-42} fibrils.

Chart 2



Polyvalency is a powerful means for designing ligands that bind more strongly to targets.[6] We have previously shown that macrocycle **1** can readily be linked to form divalent macrocyclic β-sheet structures that display two β-sheet domains.[4a] Here, we ask whether this divalency can lead to better inhibitors against Aβ aggregation. To address this question, we designed divalent linked macrocyclic β-sheets by connecting two macrocycles **1** through polyethylene glycol dicarboxylic acid (PEG diacid) linkers **3** (see Chart 2).[7] We also ask whether targeting two different hydrophobic regions of Aβ with these divalent linked macrocyclic β-sheets would lead to better activity than targeting a single hydrophobic region. To address this question, we designed homodivalent linked macrocyclic β-sheets **4** and **5** and heterodivalent linked macrocyclic β-sheets **6** (Chart 2). Homodivalent linked macrocyclic β-sheets **4** contain two copies of macrocycle **1a** containing Aβ_{17-21} (R_1–R_5 = LVFFA) linked through PEG diacid linkers, while homodivalent linked macrocyclic β-sheets **5** contain two copies of macrocycle **1b** containing Aβ_{30-34} (R_1–R_5 = AIIGL) linked through PEG diacid linkers. Heterodivalent linked macrocyclic β-sheets **6** contain one copy of macrocycle **1a** and one copy of macrocycle **1b** linked through PEG diacid linkers.

Our studies show that divalent linked macrocyclic β-sheets generally exhibit enhanced activity against Aβ aggregation and that heterodivalent linked macrocyclic β-sheets **6** are unexpectedly more active than homodivalent linked macrocyclic β-sheets **4** and **5**.

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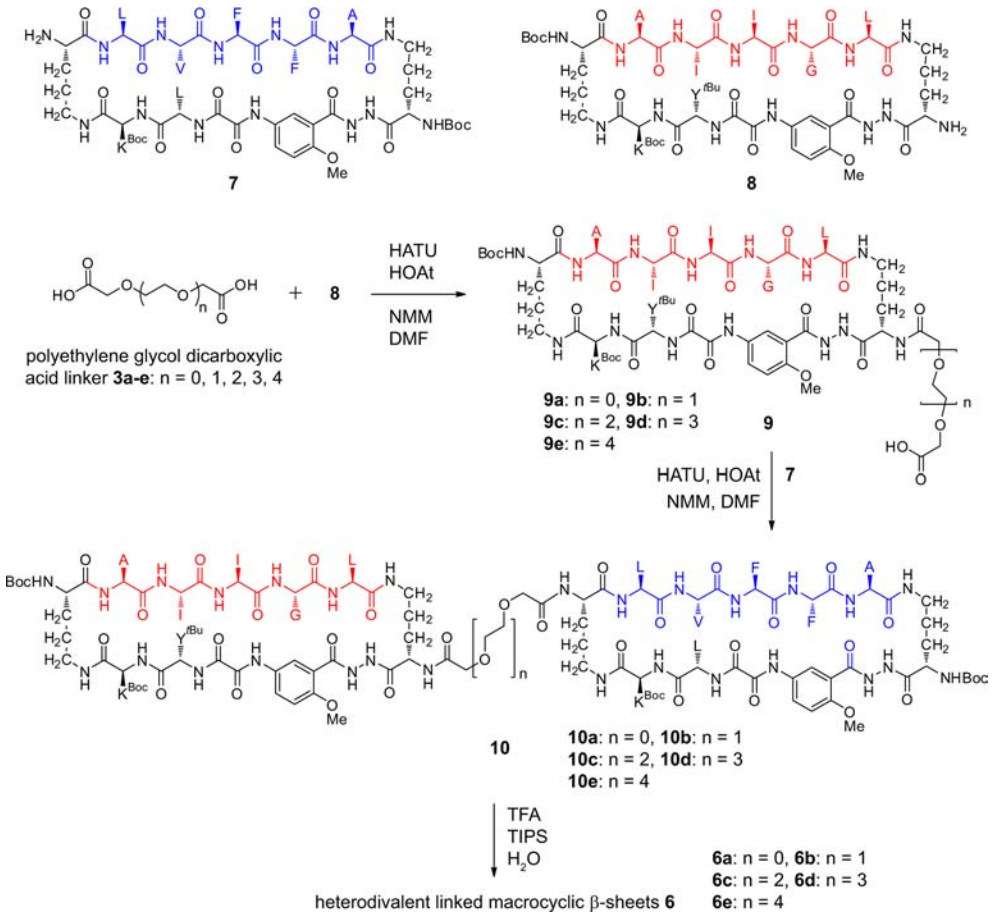
Scheme 1



RESULTS

Syntheses of Divalent Linked Macrocyclic β-Sheets 4–6. Divalent β-sheets **4**–**6** were synthesized by coupling PEG diacid linkers **3** with macrocycles **7** and **8**, which each contain a single free amino group in one of the δ-linked ornithine turn units (Scheme 1). Homodivalent β-sheets **4** and **5** were synthesized by coupling macrocycles **7** or **8** with 0.45 mol equiv of the appropriate PEG diacid linkers **3** (Scheme S1). Heterodivalent β-sheets **6a**–**e** were synthesized by first coupling macrocycle **8** with a 10-fold excess of PEG diacid linkers **3a**–**e** to give monoacids **9a**–**e** and then coupling the monoacids with macrocycle **7** (Scheme 1).

Inhibition of Aβ Aggregation by Divalent Linked Macrocyclic β-Sheets. We used thioflavin T (ThT) fluorescence assays to investigate the effects of divalent β-sheets **4**–**6** and monovalent homologues **1a** and **1b** on Aβ_{1-40} aggregation.[8] The time course of Aβ aggregation generally demonstrates a sigmoidal curve, containing a lag phase, a growth phase, and an equilibrium phase (Figure 2a). The duration of the lag phase is widely used as a diagnostic indicator of inhibition of Aβ aggregation. We thus used this lag time to evaluate the activity of **1a**, **1b**, and **4**–**6** against Aβ_{1-40} aggregation.

ThT fluorescence assays show that macrocycle **1a** containing sequence Aβ_{17-21} slightly delays Aβ_{1-40} aggregation, macrocycle **1b** containing sequence Aβ_{30-34} accelerates Aβ_{1-40} aggregation, and a mixture of 1 mol equiv of macrocycle **1a** and 1 mol equiv of macrocycle **1b** does not significantly change the lag time (Figure 2b). Macrocycle **1a** delays Aβ_{1-40} aggregation by 30% at 2 equiv, increasing the lag time from 107 to 143 min, while macrocycle **1b** accelerates Aβ_{1-40} aggregation by 50%, reducing the lag time from 107 to 57 min. A mixture of 1 equiv of macrocycle **1a** and 1 equiv of macrocycle **1b** exhibits a lag time of 106 min, which is within statistical variation of that of Aβ_{1-40} alone. These results are consistent with trends that we have observed in the effects of macrocycles **2** against Aβ aggregation and also support that the central hydrophobic sequence Aβ_{17-21} plays an important role in Aβ aggregation and in the activity of macrocycles **1** and **2** against Aβ_{1-40} aggregation.[4d,9]

ThT fluorescence assays show that heterodivalent β-sheets are not only far more active than their monovalent components but also are dramatically more active against Aβ_{1-40} aggregation than their homodivalent counterparts. Heterodivalent β-sheets **6a** and **6e** dramatically delay Aβ_{1-40} aggregation by 570% and 660% respectively at 1 equiv (15 μM), while homodivalent β-sheets **4a**, **4e**, and **5e** slightly delay aggregation by 30–70%, and homodivalent β-sheet **5a** accelerates aggregation by 40% (Figure 2c–e). Transmission electron microscopy (TEM) studies of samples taken from the ThT assays indicate that Aβ_{1-40} forms fibrils in the absence of heterodivalent β-sheet **6e** and does not form fibrils in the presence of heterodivalent β-sheet **6e** during the delayed lag time (Figure 2f).

It is interesting that there is no significant difference in lag time between heterodivalent β-sheet **6a**, which has a short linker (n = 0), and heterodivalent β-sheet **6e**, which has a longer linker (n = 4). To investigate the effect of the linker length, we synthesized additional heterodivalent β-sheets **6b**–**d**, which have linkers of intermediate length (n = 1–3). ThT

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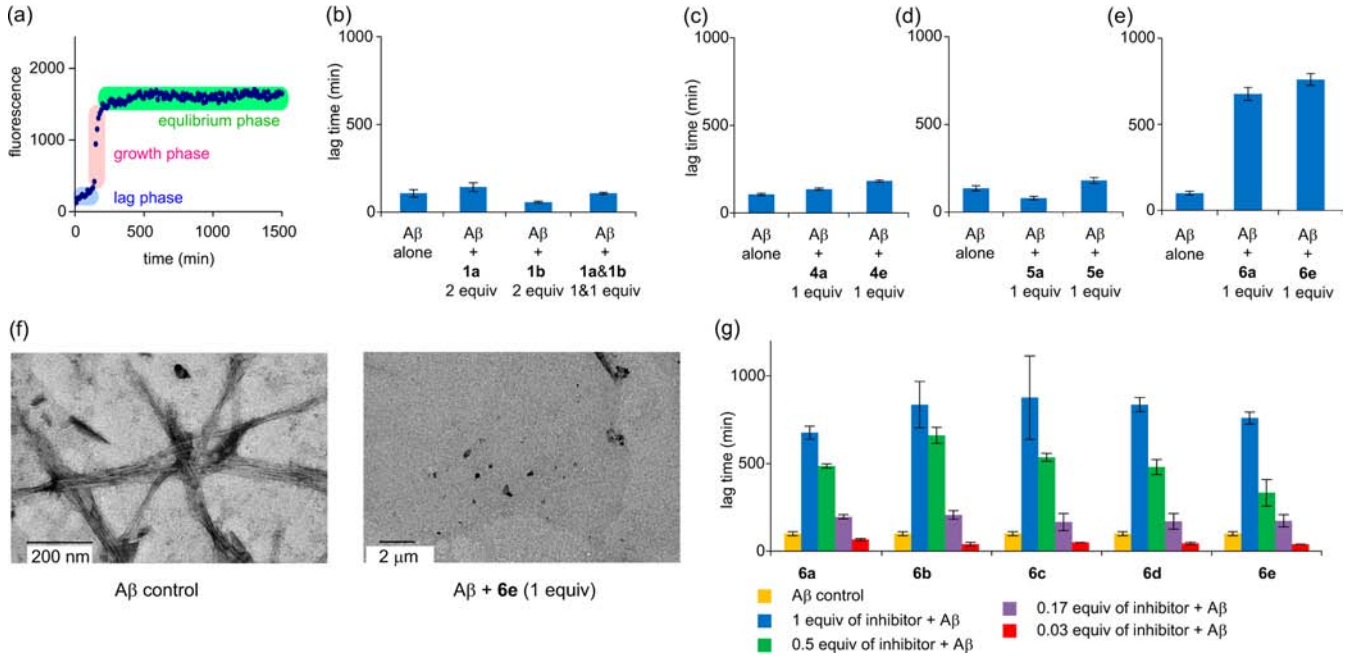

Figure 2. ThT fluorescence assays. (a) Fibrillation kinetics of Aβ_{1-40} monitored by a ThT fluorescence assay. This plot displays three phases of Aβ_{1-40} aggregation: the lag phase, the growth phase, and the equilibrium phase. (b) Lag time of Aβ_{1-40} aggregation with and without macrocycles **1a** and **1b**. (c) Lag time of Aβ_{1-40} aggregation with and without homodivalent β-sheets **4a** and **4e**. (d) Lag time of Aβ_{1-40} aggregation with and without homodivalent β-sheets **5a** and **5e**. (e) Lag time of Aβ_{1-40} aggregation with and without heterodivalent β-sheets **6a** and **6e**. (f) TEM image of Aβ_{1-40} (15 μM) after incubation for 6.5 h without (left) and with (right) heterodivalent β-sheet **6e** (1 equiv). (g) Lag time of Aβ_{1-40} aggregation with heterodivalent β-sheets **6a**–**e** at 0.03, 0.17, 0.5, and 1 mol equiv. All ThT assays were carried out on 15 μM Aβ_{1-40} in HEPES buffer at 31 °C.

fluorescence assays show that heterodivalent β-sheets **6a**–**e** delay Aβ_{1-40} aggregation by 570–770% at 1 equiv (Figure 2g). These results indicate that the size of the PEG-based diacid linkers does not substantially affect the activity of heterodivalent β-sheets **6**. ThT fluorescence assays also show that heterodivalent β-sheets **6** inhibit Aβ_{1-40} aggregation at substochiometric concentrations in a dose-dependent manner. Heterodivalent β-sheets **6a**–**e** delay Aβ_{1-40} aggregation at 0.17–1.0 equiv (2.5–15 μM) by 70–770% (Figure 2g). Surprisingly, heterodivalent β-sheets **6a**–**e** all nucleate Aβ_{1-40} aggregation at 0.03 equiv (0.5 μM), accelerating Aβ_{1-40} aggregation by 30–60%. These results indicate that both the activity and the role of the heterodivalent β-sheets in Aβ_{1-40} aggregation depend on their concentrations.[10]

DISCUSSION

It is surprising that the heterodivalent linked β-sheets show enhanced inhibitory activity, given that only one of their components inhibits Aβ_{1-40} aggregation and the other accelerates aggregation. A model based on both nucleation-dependent polymerization and that which we have previously proposed may explain this enhanced inhibition.[4d,11] In this model, Aβ_{1-40} aggregates to form early β-structured oligomers, which proceed to form a β-structured nucleus, and finally polymerize to form cross-β fibrils. Inhibitors bind to and stabilize the early β-structured oligomers and thus delay aggregation, while accelerators create a new, lower energy pathway for aggregation. Figure 3 provides a reaction free-energy diagram for the native, inhibited, and accelerated Aβ_{1-40} aggregation with black, blue, and red curves. Better inhibitors bind to the early β-structured oligomers more strongly and thus better delay the formation of the β-structured nucleus.[12]

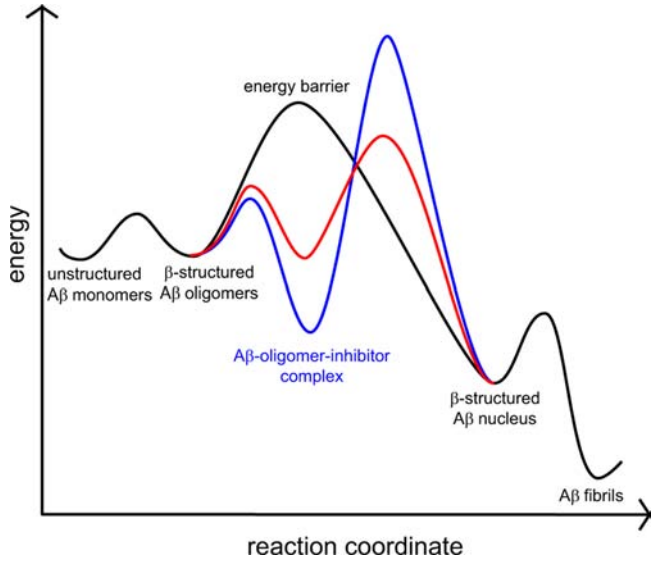

Figure 3. Effect of inhibitors and accelerators on the energetics of Aβ aggregation. The black curve corresponds to a pathway in which Aβ_{1-40} aggregates without inhibitors, while the blue and red curves correspond to pathways in which Aβ_{1-40} aggregates with inhibitors and accelerators, respectively.

The hydrophobic N-terminal Aβ_{17-21} (LVFFA) region forms the core of the β-structured oligomers, in which hydrogen bonding and hydrophobic interactions create a multilayered β-sheet structure (Figure 4a). Similar multilayered β-sheet structures are also observed in macrocycle **1a** and the amyloid-like fibrils formed by peptide fragment Aβ_{16-21} (KLVFFA).[4b,13] Macrocycle **1a** containing the N-terminal LVFFA pentapeptide complements and binds to the oligomers

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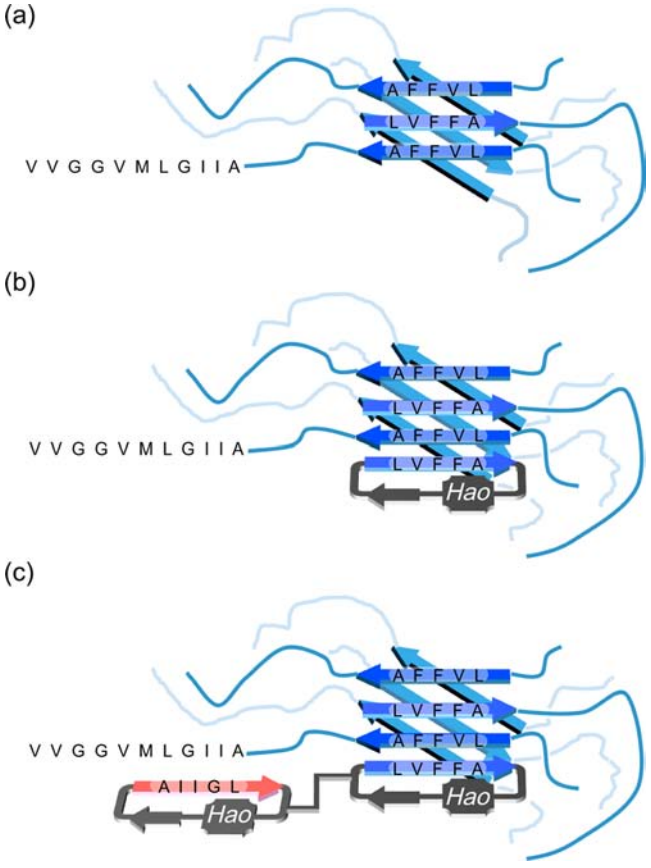

Figure 4. Model for enhanced activity of heterodivalent β-sheets **6** against Aβ_{1-40} aggregation. (a) Aβ oligomer. (b) Aβ oligomer−**1a** complex. (c) Aβ oligomer−**6** complex.

through similar types of interactions and thus inhibits aggregation (Figure 4b). Macrocycle **1b** containing the C-terminal AIIGL pentapeptide better complements the C-terminal region of Aβ_{1-40} and facilitates the transition of Aβ_{1-40} to the U-shaped superstructure associated with fibrils. In the U-shaped superstructure, the C-terminal region also forms β-sheet structure and is packed against the N-terminal region. By facilitating the formation of the U-shaped superstructure, macrocycle **1b** accelerates aggregation.

The modest effect of homodivalent linkage in **4** and **5** suggests that the β-structured oligomers do not present multiple exposed β-sheet edges in sufficient proximity to be bridged by short PEG linkers. Heterodivalent linked macrocyclic β-sheets **6** bind to the β-structured oligomers more strongly, because the LFVVA-containing macrocycle can bind to the core of the β-structured oligomers, while the AIIGL-containing macrocycle can achieve additional interactions with the C-terminal region of Aβ_{1-40} (Figure 4c). This working model may provide a framework for the design of even more effective inhibitors that target both the N- and C-terminal regions of Aβ.

CONCLUSION

The heterodivalent design of linked macrocyclic β-sheets **6** enhances their activity against Aβ aggregation. The enhanced activity suggests that polyvalent inhibitors that can target multiple regions of Aβ are better than ones that only target a single region. The strategy described herein may be applicable to design inhibitors against aggregation of other amyloid proteins.

ASSOCIATED CONTENT

Supporting Information

Details of synthesis of divalent linked macrocyclic β-sheets **4**−**6**; thioflavin T fluorescence assays of Aβ_{1-40} with **1a**, **1b**, and **4**−**6**; TEM; ESIMS and HPLC data of **4**−**6** and **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) (a) Roychaudhuri, R.; Yang, M.; Hoshi, M. M.; Teplow, D. B. *J. Biol. Chem.* **2009**, *284*, 4749−4753. (b) Jakob-Roetne, R.; Jacobsen, H. *Angew. Chem., Int. Ed.* **2009**, *48*, 3030−3059.

(2) (a) Lührs, T.; Ritter, C.; Adrian, M.; Riek-Loher, D.; Bohrmann, B.; Döbeli, H.; Schubert, D.; Riek, R. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 17342−17347. (b) Petkova, A. T.; Yau, W.-M.; Tycko, R. *Biochemistry* **2006**, *45*, 498−512.

(3) (a) Luca, S.; Yau, W.-M.; Leapman, R.; Tycko, R. *Biochemistry* **2007**, *46*, 13505−13522. (b) Hebda, J. A.; Miranker, A. D. *Annu. Rev. Biophys.* **2009**, *38*, 125−152. (c) Wiltzius, J. J. W.; Sievers, S. A.; Sawaya, M. R.; Eisenberg, D. *Protein Sci.* **2009**, *18*, 1521−1530. (d) Middleton, C. T.; Marek, P.; Cao, P.; Chiu, C.-C.; Singh, S.; Woys, A. M.; de Pablo, J. J.; Raleigh, D. P.; Zanni, M. T. *Nat. Chem.* **2012**, *4*, 355−360.

(4) (a) Woods, R. J.; Brower, J. O.; Castellanos, E.; Hashemzadeh, M.; Khakshoor, O.; Russu, W. A.; Nowick, J. S. *J. Am. Chem. Soc.* **2007**, *129*, 2548−2558. (b) Liu, C.; Sawaya, M. R.; Cheng, P.-N.; Zheng, J.; Nowick, J. S.; Eisenberg, D. *J. Am. Chem. Soc.* **2011**, *133*, 6736−6744. (c) Zheng, J.; Liu, C.; Sawaya, M. R.; Vadla, B.; Khan, S.; Woods, R. J.; Eisenberg, D.; Goux, W. J.; Nowick, J. S. *J. Am. Chem. Soc.* **2011**, *133*, 3144−3157. (d) Cheng, P.-N.; Liu, C.; Zhao, M.; Eisenberg, D.; Nowick, J. S. *Nat. Chem.* **2012**, in press.

(5) (a) von Bergen, M.; Friedhoff, P.; Biernat, J.; Heberle, J.; Mandelkow, E. M.; Mandelkow, E. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5129−5134. (b) Inouye, H.; Sharma, D.; Goux, W. J.; Kirschner, D. A. *Biophys. J.* **2006**, *90*, 1774−1789.

(6) (a) Mammen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754−2794. (b) Kim, Y.-S.; Lee, J.-H.; Ryu, J.; Kim, D.-J. *Curr. Pharm. Des.* **2009**, *15*, 637−658.

(7) (a) Zutshi, R.; Shultz, M. D.; Ulysse, L.; Lutgring, R.; Bishop, P.; Schweitzer, B.; Vogel, K.; Franciskovich, J.; Wilson, M.; Chmielewski, J. *Synlett* **1998**, 1040−1044. (b) Wittmann, V.; Takayama, S.; Gong, K. W.; Weitz-Schmidt, G.; Wong, C.-H. *J. Org. Chem.* **1998**, *63*, 5137−5143.

(8) LeVine, H. *Methods Enzymol.* **1999**, *309*, 274−284.

(9) (a) Tjernberg, L. O.; Näslund, J.; Lindqvist, F.; Johansson, J.; Karlström, A. R.; Thyberg, J.; Terenius, L.; Nordstedt, C. *J. Biol. Chem.* **1996**, *271*, 8545−8548. (b) Sciarretta, K. L.; Gordon, D. J.; Meredith, S. C. *Methods Enzymol.* **2006**, *413*, 273−312. (c) Williams, A. D.;

Shivaprasad, S.; Wetzel, R. *J. Mol. Biol.* **2006**, *357*, 1283−1294. (d) Estrada, L. D.; Soto, C. *Curr. Top. Med. Chem.* **2007**, *7*, 115−126. (e) Miller, Y.; Ma, B.; Nussinov, R. *Chem. Rev.* **2010**, *110*, 4820−4838.

(10) The acceleration of $A\beta_{1-40}$ aggregation at low concentrations of heterodivalent β-sheets **6a−e** suggests that **6a−e** may accelerate aggregation in monomeric form and inhibit aggregation in oligomeric form.

(11) Finder, V. H.; Glockshuber, R. *Neurodegener. Dis.* **2007**, *4*, 13−27.

(12) An alternative model for the inhibition involves binding of the heterodivalent inhibitors to the N- and C-terminal β-sheet regions of small Aβ fibrils and thus the prevention of their elongation by a *capping* mechanism. The observation that even inhibitors with very short linkers (e.g., **6a**, $n = 0$) block aggregation does not appear to be consistent with this alternative model, because the separation of the N- and C-terminal β-sheet regions of the Aβ fibrils is larger than the linker (ref 2b).

(13) Colletier, J.-P.; Laganowsky, A.; Landau, M.; Zhao, M.; Soriaga, A. B.; Goldschmidt, L.; Flot, D.; Cascio, D.; Sawaya, M. R.; Eisenberg, D. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 16938−16943.