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Bivalent Ligands Targeting Multiple Pathological Factors Involved in Alzheimer's Disease

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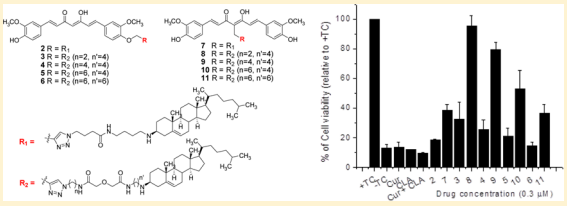
Supporting Information

ABSTRACT: In a continuing effort to develop multifunctional compounds as potential treatment agents for Alzheimer's disease (AD), a series of bivalent ligands containing curcumin and cholesterylamine were designed, synthesized, and biologically characterized. Biological characterization supported earlier results that the spacer length and its attachment position on curcumin are essential structural determinants for biological activity in this class. Compounds with a spacer length of 17−21 atoms exhibited optimal neuroprotection in human neuroblastoma MC65 cells with submicromolar potency. These compounds inhibited the formation of amyloid-β oligomers (AβOs) and exhibited antioxidative activities in MC65 cells. Bivalent ligand **8**, with its spacer (length of 17 atoms) connected at the methylene carbon between the two carbonyls of the curcumin moiety, is the most potent with an EC_{50} of 0.083 ± 0.017 μM. In addition, **8** formed a complex with biometals, such as Cu, Fe, and Zn. Collectively, the results strongly support our assertion that these compounds are designed bivalent ligands with potential as multifunctional and neuroprotective agents.



KEYWORDS: *bivalent ligands, multifunctional, Aβ oligomers, antioxidant, metal chelating, Alzheimer's disease*

It is estimated that 5.4 million Americans of all ages are affected by Alzheimer's disease (AD), which is devastatingly neurodegenerative and the most common cause of dementia. In addition to human cost, more than $200 billion is spent annually on AD treatment, significantly exacerbating problems with the overextended U.S. health care economy.[1] Multiple pathological risk factors have been suggested to contribute to AD, and these include misfolded β-amyloid (Aβ), phosphorylated τ, dyshomeostasis of biometals, oxidative stress (OS), and neuroinflammation, among others.[2−6] With the ongoing progess in understanding basic mechanisms leading to AD, numerous chemical ligands targeting the proposed risk factors, such as secretase inhibitors,[7,8] Aβ oligomerization/aggregation inhibitors,[9−14] metal-complexing agents,[15] antioxidants, and anti-inflammatory agents,[16] have been developed and tested in the past decade as potential AD-modifying agents. Unfortunately, none of these proposed disease-modifying agents have been approved by the FDA, which suggests limitations in the traditional "one molecule, one target" paradigm. This also suggests that it is not sufficient to develop effective AD pharmacotherapy by targeting a single risk factor, especially given the complex nature of this disease. Consequently, these difficulties present an opportunity by requiring the more efficient ways to treat AD by, for example, cotargeting these factors with a single pleiotropic molecule.

Recently, we have developed a bivalent strategy for the design of multifunctional ligands by incorporating cell membrane/lipid rafts (CM/LR) anchorage into molecular design,[17] since CM/LR have been implicated as a critical platform in facilitating Aβ oligomerization, OS, and subsequent neurotoxicity.[18−20] Our results demonstrated that these bivalent ligands can localize to the CM/LR, and neuroprotection efficiency can be significantly improved by the resulting CM/LR anchorage.[17] Furthermore, these bivalent ligands can penetrate the blood−brain barrier and function as novel fluorescence probes for Aβ plaques in the brain.[21] To further validate this strategy and develop more potent analogues, we report here the design and biological characterization of a new series of bivalent ligands containing curcumin and cholesterylamine (CLA) for their neuroprotective, antioxidative, and metal-chelating activities.

In a previous report, we presented the design of bivalent ligands containing curcumin as the multifunctional warhead and cholesterol as the CM/LR anchor.[17] Characterization of the first generation bivalent ligands proved that our novel molecular design produced bivalent ligands that anchored to CM/LR and exhibited multifunctional properties. The reports that *N*-alkyl derivatives of CLA can also effectively anchor CM/LR in mammalian cells and function as carriers via endocytosis with improved activity over cholesterol[22−25] suggested a new series of bivalent ligands with CLA as the CM/LR anchor. An additional consideration is that replacement of cholesterol with CLA alleviates potential concerns regarding the introduction of additional cholesterol into the body. The new series of bivalent

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ligands (2−11) with CLA as CM/LR anchor were designed based on the lead bivalent ligand **1** to investigate whether NH is preferred over O in neuroprotection and whether spacer connectivity on curcumin is still critical in this series of bivalent ligands (Figure 1). The spacer length of 2−11 varies in this series from 13 to 23 atoms to evaluate optimal spacer length and for comparison with that of our first generation bivalent compounds.

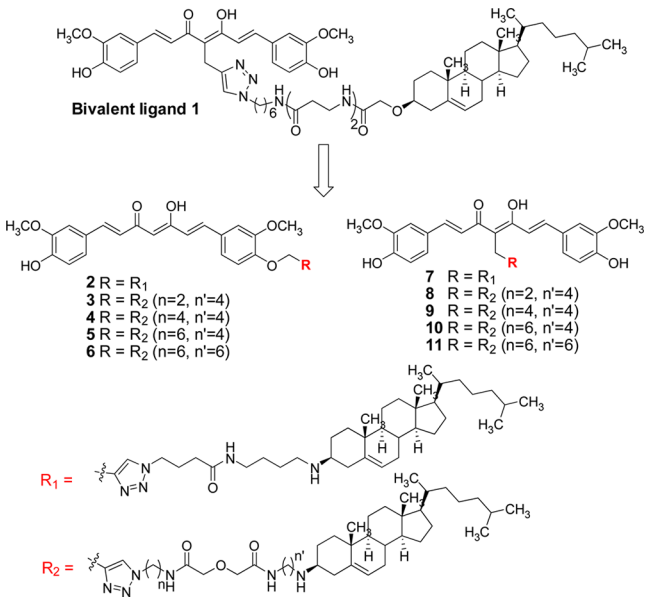
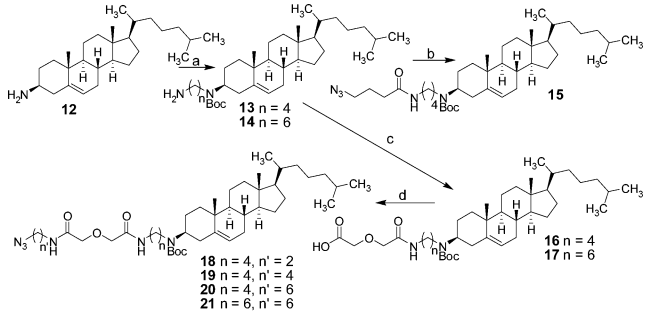


Figure 1. Lead bivalent ligand **1** and newly designed bivalent ligands.

The synthesis of compounds **2−11** is outlined in Schemes 1 and 2. Briefly, intermediates **13** and **14** were prepared by

Scheme 1. Synthesis of Intermediates 15 and 18−21[a]

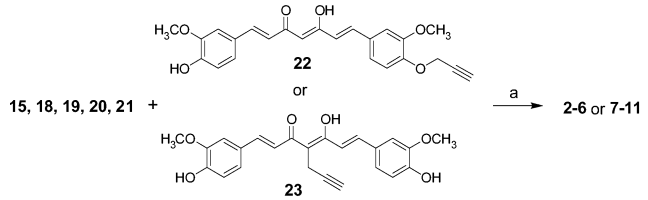


[a]Reagents and conditions: a) i. N-(4-bromoalkyl)phthalimide, K_2CO_3, DMF, 60 °C; ii. $(Boc)_2O$, DIPEA, DCM; iii. Hydrazine, EtOH, reflux; b) HOBt, EDC, 4-azidobutyric acid, DCM; c) diglycolic anhydride, TEA, DCM; d) azidoalkylamine, EDC, DIPEA, DCM.

[a]Reagents and conditions: (a) (i) *N*-(4-Bromoalkyl)phthalimide, K_2CO_3, DMF, 60 °C; (ii) $(Boc)_2O$, DIPEA, DCM; (iii) hydrazine, EtOH, reflux. (b) HOBt, EDC, 4-azidobutyric acid, DCM. (c) Diglycolic anhydride, TEA, DCM. (d) Azidoalkylamine, EDC, DIPEA, DCM.

reacting of 3β-CLA 12 with *N*-(4-bromo-butyl)-phthalimide or *N*-(6-bromo-hexanyl)-phthalimide followed by Boc protection and removal of the phthaloyl group using hydrazine.[26] The coupling reaction of **13** with 4-azido-butyric acid afforded intermediate **15**. Reactions of diglycolic anhydride with **13** or **14** yielded **16** and **17**, respectively, that, after coupling reactions with various azidoamines, afforded intermediates **18−21** with

Scheme 2. Synthesis of Target Bivalent Ligands 2−11[a]



[a]Reagents and conditions: (a) (i) Sodium ascorbate, $CuSO_4$, THF/H_2O (1/1); (ii) TFA, DCM.

good yields (Scheme 1). As shown in Scheme 2, the click reactions of the alkynes **22** or **23**, prepared as we previously reported,[17] with **15** and **18−21** were applied under sodium ascorbate and $CuSO_4$ in tetrahydrofuran (THF)/H_2O conditions to obtain the designed bivalent ligands **2−11**. All bivalent ligands are in keto−enol forms in chloroform as determined by ^{1}H NMR and ^{13}C NMR.

MC65 is a human neuroblastoma cell line that conditionally expresses C99, the C terminus fragment of APP using tetracycline (TC) as transgene suppressor.[27] Upon removal of TC, these cells can produce intracellular Aβ aggregates including small amyloid-β oligomers (AβOs). In our previous studies, human neuroblastoma MC65 cells were demonstrated to be good models to test our multifunctional ligands.[17] Therefore, after synthesis, we first evaluated the neuroprotective activities of bivalent ligands **2−11** in MC65 cells under TC removal conditions. For initial screening, we chose to test the compounds at a concentration of 0.3 μM since we are trying to develop new bivalent ligands with potency in the submicromolar range, with dose−response studies to follow for the more active ligands. As shown in Figure 2A, upon removal of TC, the viability of MC65 cells was significantly reduced (13% as compared to 100% TC control). In general, bivalent ligands with the spacer connection at the methylene carbon of curcumin (**7−11**) exhibited better neuroprotective activity as compared to those with the spacer connection at the phenoxyl oxygen of curcumin (**2−6**) (See Figure 1). These results are fully consistent with our previous report for the cholesterol-containing first generation analogues.[17] The replacement of cholesterol with CLA does not influence preferred locus for spacer connectivity. Notably, bivalent ligand **8** with a 17-atom spacer exhibited the most potent neuroprotection at 0.3 μM concentration in MC65 cells and both decreases and increases in spacer length led to a decrease in neuroprotective activity under the experimental conditions. Interestingly, the same pattern was also noticed in compounds **2−6**, with **3** having the peak activity. Clearly, consistent with our previous report, spacer length is critical to biological activity with an optimal spacer range within the **7−11** series of 17−21 atoms, which is slightly different from the optimal spacer range of the cholesterol-containing ligands where a 21-atom spacer is most potent. This might indicate that the change from cholesterol to CLA may yield different interactions of the ligands with CM/LR, thus changing the general orientation of the ligands and thereby affecting the overall neuroprotective activity. Further studies are being undertaken to confirm this hypothesis.

After identification of active ligands at 0.3 μM, dose−response studies were conducted for bivalent ligands **8−10** in MC65 cells to obtain neuroprotective EC_{50} values. As shown in Figure 2B, the dose−response studies revealed EC_{50} values of 0.083 ± 0.017, 0.16 ± 0.026, and 0.30 ± 0.082 μM for **8−10**,

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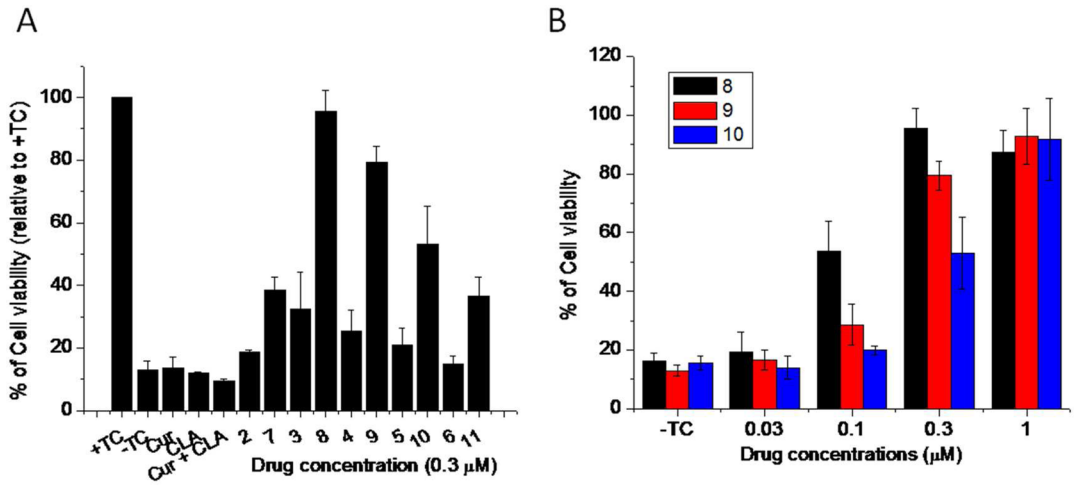

Figure 2. Neuroprotective effects on MC65 cells. (A) MC65 cells were treated with indicated compounds at 0.3 μM under +TC or −TC conditions for 72 h. Cell viability was assayed by MTT assay. (B) MC65 cells were treated with indicated compounds at indicated concentrations under +TC or −TC conditions for 72 h. Cell viability was assayed by MTT assay. Data were expressed as mean percentage viability (n = 3) with parallel +TC cultures set at 100% viability. Error bars represent the SEM.

respectively. The results also clearly indicated that neuroprotective potency of CLA-containing series of bivalent ligands is significantly better than that of the cholesterol-containing series (EC_{50} = 3 μM for the best ligand **1**), which supports our hypothesis that structural modifications in the CM/LR anchor domain can provide more potent analogues.

Under TC removal conditions, MC65 cells can produce intracellular Aβ aggregates including small Aβ oligomers (AβOs), and the induced cytotoxicity in these cells by TC removal has been associated with the accumulation of AβOs.[27] To obtain preliminary molecular mechanisms underlying the neuroprotective activity of these bivalent ligands, we next evaluated the inhibitory effects of the most active compounds, **8**−**10**, on the production of AβOs. As shown in Figure 3A, all

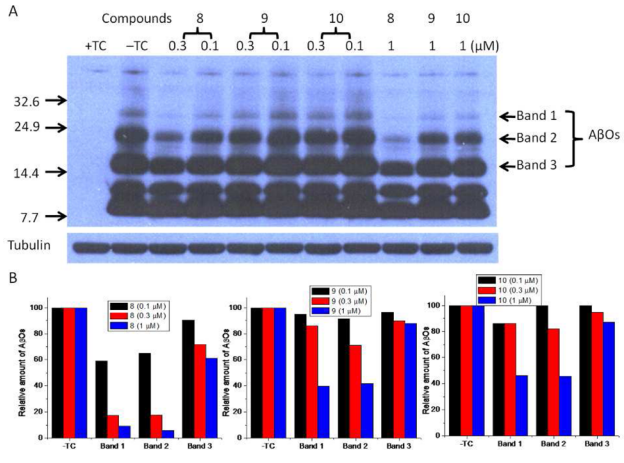

Figure 3. Inhibition of AβO formation by 8, 9, and 10 in MC65 cells. (A) MC65 cells were treated with indicated compounds at indicated concentrations for 30 h immediately after the removal of TC. Lysates from cultures were analyzed by Western blotting using 6E10 antibody. The image represents the results from one of two independent experiments. (B) The relative amount of band 1, band 2, and band 3 in the immunoblot quantified by densitometry and normalized as a fraction of the α-tubulin levels. The −TC control to tubulin ratio was set at 100%, and the AβO values for the treatment conditions were expressed as a percentage of the −TC control.

three compounds inhibited the production of small AβOs, such as tetramers, pentamers, and heptamers in MC65 cells, with **8** being the most potent one, consistent with the MC65 neuroprotection assay results. Quantification of AβOs by densitometry confirmed that all three compounds dose dependently reduced the production of AβOs (Figure 3B). However, as compared to the neuroprotection results, the inhibitory activity of AβO production is somewhat weaker for the tested compounds. This may suggest that inhibition of AβO production might be only one of the contributing factors toward the overall neuroprotection outcomes; that is, these ligands may manifest significant neuroprotection for MC65 cells through synergistic and/or additive effects of multiple mechanisms, even while exhibiting less potency for a single (measurable) factor. This is, in effect, indirect but key support for our design rationale of multifunctional ligands as potential AD-modifying agents. The C-99 fragment has shown Aβ-independent neurotoxicity;[28] however, we did not observe any significant effects of these compounds on the expression of C99 (data not shown) under the experimental conditions.

One of our design goals for these bivalent ligands is to reduce OS that potentially contributes to the development of AD. Furthermore, OS has been suggested to impart neurotoxicity upon the accumulation of intracellular AβOs in MC65 cells.[29] Therefore, we evaluated the antioxidative activity of **8**−**10** in MC65 cells using the dichlorofluorescein diacetate (DCFH-DA) assay to investigate whether antioxidation is one of the mechanisms that lead to neuroprotection of these bivalent ligands. As shown in Figure 4A, TC removal led to extensive accumulation of intracellular OS in MC65 cells (~46% increase in fluorescence intensity as compared to +TC control). Notably, **8** significantly suppressed the intracellular OS by 35% at as low as 0.1 μM concentration, and the suppression of intracellular OS is dose-depndent. Bivalent ligand **9** exhibited comparable antioxidative activity to **8**, while compound **10** was slightly less potent.

Lastly, we studied the metal complexation of bivalent ligand **8** by UV−vis spectroscopy since curcumin has been demonstrated to chelate biometals[30] and dyshomeostasis of metal ions has been indicated as one of the pathologies of AD.[31] In addition, it has been suggested that metal ions are

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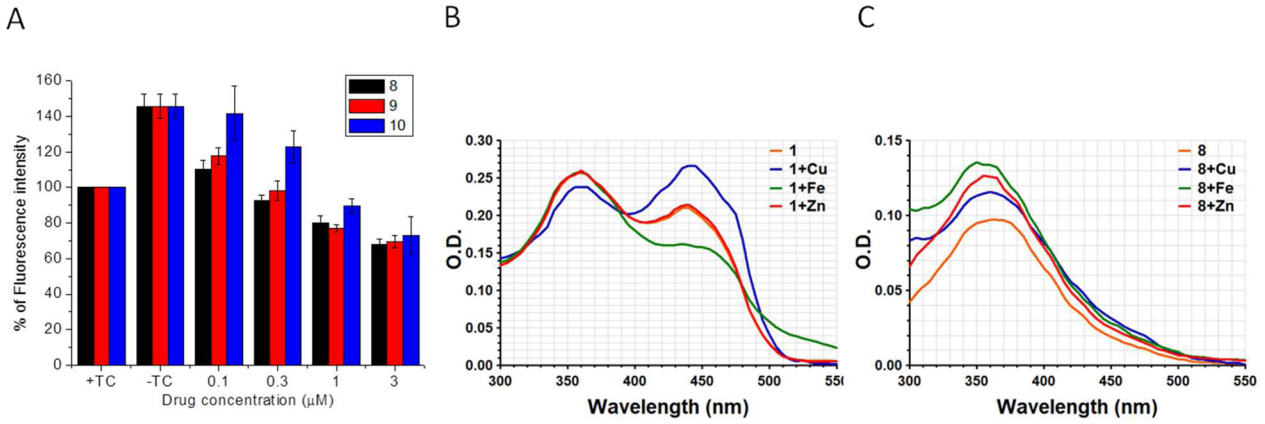

Figure 4. Antioxidant and metal chelating properties. (A) MC65 cells were treated with indicated compounds at indicated concentrations under +TC or −TC conditions for 48 h, then DCFH-DA (25 μM) was loaded, and the fluorescence intensity of the cells was analyzed by flowcytometry. Data were presented as a mean percentage of fluorescence intensity (n = 3) with parallel +TC cultures set at 100%. Error bars represent SEMs. (B and C) Indicated compound (50 μM) was incubated with $CuSO_4$, $FeCl_2$, or $ZnCl_2$ (60 μM), respectively, at room temperature for 10 min, and then, the UV−vis spectrum was recorded from 300 to 550 nm.

involved in the assembly and neurotoxicity of Aβ.[32] Therefore, a compound with metal complxation properties may add another layer of benefits in developing multifunctional ligands as AD-modifying agents. Bivalent ligand **1** was tested as a control and comparison. As shown in Figure 4B, ligand **1** showed maximum absorption at 360 and 440 nm in the absence of metal ions, but when metal ions (Cu^{2+}, Fe^{2+}, or Zn^{2+}) were added, the spectra showed the formation of **1**−Cu^{2+} and **1**−Fe^{2+} complexes, with no appreciable complexation between **1** and Zn^{2+}. These observations are consistent with the reported results of curcumin itself.[29] In contrast, as shown in Figure 4C, under the same experimental conditions, **8** showed an absorption maximum at 365 nm in the absence of metal ions. When metal ions (Cu^{2+}, Fe^{2+}, or Zn^{2+}) were added to **8**, the spectra revealed increase in optical intensity and blue shifts of the absorption maximum for all three metal ions, that is, **8** complexes with Cu^{2+}, Fe^{2+}, and Zn^{2+}. We believe that this indicates that more groups of the molecule, including the CM/LR anchor (such as the NH in CLA) and/or the spacer, is involved in complexation rather just the curcumin moiety. These results are further support for our development of alternatives to the cholesterol-anchored series can provide new analogues with novel pharmacological activity and potency.

In summary, we have developed a new series of bivalent ligands containing curcumin and CLA to further explore our strategy for developing bivalent multifunctional ligands as AD-modifying agents. Biological characterization demonstrated that spacer length and connectivity loci on the curcumin moiety are critical structural features with respect to their biological activities. Among the reported ligands, **8** with a 17-atom spacer exhibited the best neuroprotection on MC65 cells with an EC_{50} of 0.083 ± 0.017 μM. The neuroprotective activity may be due to its anti-AβO, antioxidation, and metal-chelating properties, all of which were exhibited by this compound. Collectively, the results from this study suggest that the bivalent multifunctional strategy is valid and holds promise in developing effective AD treatments. Further development and optimization of **8** as a lead compound may provide even more potent analogues.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details for synthetic procedures, analytical data, and biological studies for compounds **1**−**11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Chemical synthesis and structural characterization were completed by K.L., J.C., and R.G. In vitro studies using MC65 cells and metal complexation studies were performed by K.L. Experiment design, data analysis, writing, and editing were completed by S.Z.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Aβ, amyloid-β; AβOs, amyloid-β oligomers; AD, Alzheimer's disease; CLA, cholesterylamine; CM/LR, cell membrane/lipid rafts; DCFH-DA, dichlorofluorescein diacetate; OS, oxidative stress; TC, tetracycline; THF, tetrahydrofuran; DMF, *N*,*N*-dimethylformamide; DIPEA, *N*,*N*-diisopropylethylamine; DCM, dichloromethane; HOBt, 1-hydroxybenzotriazole; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; TEA, triethylamine; TFA, trifluoroacetic acid

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