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Development of bivalent compounds as potential neuroprotectants for Alzheimer's disease

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Abstract

In our efforts to further investigate the impact of the spacer and membrane anchor to the neuroprotective activities, a series of bivalent compounds that contain cholesterol and extended spacers were designed, synthesized and biologically characterized. Our results support previous studies that incorporation of a piperazine ring into the spacer significantly improved the protective potency of bivalent compounds in MC65 cell model. Spacer length beyond 21 atoms does not add further benefits with **21MO** being the most potent one with an EC₅₀ of 81.86 ± 11.91 nM. Our results also demonstrated that bivalent compound **21MO** suppressed the production of mitochondria reactive oxygen species. Furthermore, our results confirmed that both of the spacer and membrane anchor moiety are essential to metal binding. Collectively, the results provide further evidence and information to guide optimization of such bivalent compounds as potential neuroprotectants for Alzheimer's disease.

Keywords

Alzheimer's disease; Bivalent compounds; Rational drug design; Medicinal chemistry; SAR

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that leads to deterioration of cognitive and functional abilities. AD is also the leading cause of dementia affecting ~5.5 million Americans.¹ Currently available medications can only provide symptomatic relief for AD patients and no agent is available to cure or delay this disease. The elusive etiology of AD is a confounding factor: multiple pathogenic factors such as aggregates of beta-amyloid (A β) and tau protein, oxidative stress, neuroinflammation, and mitochondrial dysfunction, among others, have been implicated in the development of AD.^{2–9} Although significant advances have been made in understanding the pathology of this devastating disease, our community is still anxiously waiting for the success of a variety of clinical trials based on strategies targeting various risk factors.

Based on the multifactorial nature of AD, we have developed a bivalent strategy to incorporate multifunctional properties into the molecules.^{10–17} Biological characterization of the designed bivalent compounds in a cellular AD model using neuronal MC65 cells

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Appendix A. Supplementary data

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demonstrated that 1) bivalent compounds are superior than either of the two individual pharmacophores or the combination of these two in protecting MC65 cells from tetracycline (TC) withdrawal induced cytotoxicity; 2) membrane anchoring moiety is critical for the observed neuroprotective activities; 3) the spacer that tethers the two pharmacophores is essential with the optimal spacer length being from 17 to 21 atoms. Our recent studies of bivalent compounds containing cholesterylamine as the membrane anchoring moiety suggested that incorporation of a piperazine ring into the spacer significantly increased the protective potency and spacer extension beyond 21 atoms provides no further benefits.¹⁰ Notably, the metal chelating capacity was diminished in this series of bivalent compounds. To further investigate whether such observations will be translated into bivalent compounds with a different membrane anchoring moiety, herein, we report the synthesis and biological characterization of a new series of bivalent compounds containing cholesterol as the membrane anchor.

To directly compare with the results of our previously reported bivalent compounds that contain cholesterylamine as membrane anchor and to minimize the variation of spacer, we employed the same spacer that contains a piperazine ring in the newly designed bivalent compounds (Fig. 1). The spacer length ranges from 21 to 39 atoms with an increment of 2 atoms each time, same as we did for the cholesterylamine containing bivalent compounds. For the compound coding, the Arabic numeral represents the number of atoms in the spacer; the “M” indicates the middle methylene position between the two carbonyl groups of curcumin; and the “O” indicates the connection of the spacer to the anchoring moiety (cholesterol) is through an oxygen. The chemical syntheses for the designed bivalent compounds were accomplished following the procedures that were used to construct the cholesterylamine-containing bivalent compounds as outlined in Schemes 1–3.¹⁰

After chemical synthesis, we evaluated their protective potencies in neuronal MC65 cells, a well-established cellular AD model that involves multiple known risk factors such as A β oligomers, oxidative stress, calcium dyshomeostasis, and mitochondrial dysfunction.^{18–20} MC65 is a neuronal cell line that conditionally expresses C99, the C-terminus fragment of amyloid precursor protein (APP) using tetracycline (TC) as transgene suppressor.^{18,20} Upon removal of TC, MC65 cells produce intracellular A β aggregates including small A β oligomers (A β O5). More importantly, the induced cytotoxicity in these cells by TC removal has been associated with the accumulation of A β O5 and oxidative stress, two of the well studied risk factors in AD development. Studies from our group and others have demonstrated that this cellular assay is a suitable screening model to test small molecule compounds with potential protective activities.^{15,17,21–24} As shown in Table 1, in general, increase of the spacer length beyond 21 atoms did not lead to improvements in protective potency in this cellular model. This is consistent with the results from our previous studies of bivalent compounds containing cholesterylamine as the membrane anchor.¹⁰ Among the tested bivalent compounds, **21MO** shows the most potent protection with an EC₅₀ of 81.86 nM. When compared to a similar bivalent compound with a spacer length of 21 atoms yet different composition,¹¹ the protective potency is increased by 23 fold (81.86 vs 1848.66 nM, respectively). This further demonstrated that incorporation of a piperazine ring into the spacer significantly improves potency and spacer composition is important for the molecular

design of such bivalent compounds. Bivalent compound **35MO** shows the least protective potency with an EC_{50} of 359.48 nM, ~4-fold decrease compared to that of **21MO**. This might be due to the involvement of intrinsic cytotoxicity of this compound as suggested by our previous studies.¹⁰ A close examination of the dose-response curve of this series of bivalent compounds reveals that the observed protection of MC65 cells by the bivalent compounds at 1.0 μ M is significantly decreased when the spacer length is beyond 29 atoms, while at a higher concentration of 3.0 μ M, the protection steadily decreases with the increasing spacer length (Fig. 2A and Fig. S1). Our previous studies of cholesterylamine containing bivalent compounds demonstrated intrinsic cytotoxicity when the spacer length is > 29 atoms.^{10,13} The results from the current series of compounds further confirmed this notion. When compared to previously reported bivalent compounds with different membrane anchors,¹⁰ the current compounds exhibit reduced protective potency in general, thus confirming the essential role of the anchoring moiety on the observed protective activities.

When oxidative stress level was measured in the MC65 cell model, removal of TC led to a significant increase of cellular oxidative stress as reflected by the cellular fluorescence intensity of dichlorofluorescein diacetate (DCFH-DA) (Fig. 2B).^{18,25} Overall, treatment of MC65 cells with bivalent compounds (1.0 μ M) suppressed the accumulation of intracellular oxidative stress. Our previous studies demonstrated that mitochondrial reactive oxygen species (mitoROS) is linked to the observed cell death upon TC removal in this MC65 cell model.^{12,15,26} Our studies also suggested that bivalent compounds can localize to mitochondria, depending upon the nature of spacer and membrane anchor.^{12,14} So we next examined the effects of **21MO** treatment on the level of mitoROS. As shown in Fig. 2C, upon removal of TC, there is a significant production of mitoROS as reflected by the change of mitoSOX fluorescence. Notably, **21MO** dose dependently suppressed the TC removal induced mitoROS. This is consistent with our previous studies,^{12,14} suggesting that suppression of mitoROS by the bivalent compounds could be the contributing mechanism for the observed protection in MC65 cells.

Recently, our studies suggested that the presence of a piperazine ring in the spacer of bivalent compounds could affect the metal chelating capacity of these compounds.¹⁰ Our previous studies also suggested that the membrane anchor could contribute to the metal chelating ability of these bivalent compounds.¹⁰ Since the current series of bivalent compounds contain a similar spacer composition as the one in the cholesterylamine containing bivalent compounds, we studied the metal chelating properties of these bivalent compounds by UV-Vis spectroscopy to further confirm the roles of the membrane anchor in such property using **21MO** and **29MO** as representative compounds. As shown in Fig. 3, both compounds showed maximum absorption around 360 and 440 nm wavelength in the absence of metal ions. When metal ions (Cu^{2+} , Fe^{2+} , and Zn^{2+}) were added, the absorption spectra suggested the formation of complexes with Cu^{2+} and Fe^{2+} , but not with Zn^{2+} , by the bivalent compounds. The results are consistent with the reported results of curcumin and our cholesterol containing bivalent compound.^{15,18} Our previous studies demonstrated that diosgenin containing bivalent compounds don't chelate with any of these three ions while cholesterylamine containing bivalent compounds form complexes with all three ions.¹³ Taken together, the results again confirmed that the membrane anchoring moiety of the

bivalent compounds is critical to determine their metal chelating capacity with cholesterol being recognized by only Cu^{2+} and Fe^{2+} . If metal binding is an important feature in molecule design, both spacer composition and the membrane anchoring moiety need to be considered in the future design of such bivalent compounds.

In summary, a series of bivalent compounds with cholesterol as the membrane anchoring moiety and extended spacers were designed to further validate the contribution of the spacer and membrane anchor to the neuroprotective activities. The results established that incorporation of a piperazine ring into the spacer significantly improves the protective potency in the MC65 cellular model. This might be due to the conformation constrain of bivalent compounds conferred by this ring structure. The results further validated that the optimal spacer length of such bivalent compound is around 21 atoms and any extension beyond this length does not improve protective potency. Studies of metal chelating further confirmed the important roles of the membrane anchoring moiety within the bivalent structure to determine the interactions with metal ions. Collectively, the results from this series of bivalent compounds confirm and provide valuable guidance for future design and development of such bivalent compounds as potential treatments for AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1.
Chemical structures of designed bivalents compounds.

Fig. 2.

(A) Neuroprotective effects on MC65 cells. MC65 cells were treated with indicated compounds at 3 μ M and 1 μ M 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 + TC cultures set at 100% viability. Error bars represent the SEM. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to + TC control by student t -test); (B) MC65 cells were treated indicated compounds (1 μ M) for 48 h. Cells then were incubated with DCFH-DA (25 μ M) for 1 h. The level of ROS is represented as percentage of fluorescence intensity compared to the + TC control. Error bars represent the SEM. (* $p < 0.05$, ** $p < 0.01$ compared to –TC control by student t -test). (C) MC65 cells were treated with **21MO** (1, 0.3, 0.1 μ M) for 48 h. Cells then were incubated with MitoSOX (2.5 μ M) for 30 min. The amount of mitochondrial ROS is represented as percent fluorescence intensity compared to the + TC control. Error bars represent the SEM. (* $p < 0.05$, compared to –TC control by student t -test).

Fig. 3. Metal chelating of **21MO** and **29MO**. Indicated compound (50 μ M) was incubated with CuSO_4 , FeCl_2 , or ZnCl_2 (60 μ M), respectively, at room temperature for 10 min, and then, the UV-vis spectrum was recorded from 300 to 550 nm.

Scheme 1.

Reagents and conditions: (a) HOBt, EDCI, DCM. (b) TFA, DCM. (c) azido-carboxylic acids, HOBt, EDCI, DCM. (d) Sodium ascorbate, CuSO₄, H₂O/THF (1:1).

Scheme 2.

Reagents and conditions: (a) azido-carboxylic acids, sodium ascorbate, CuSO_4 , $\text{H}_2\text{O}/\text{THF}$ (1:1). (b) NaH , THF/DMF (100:1), 50°C . (c) *tert*-butyl (3-(4-(3-aminopropyl)piperazin-1-yl) propyl)carbamate, HOBt, EDCI, DCM. (d) 4M HCl, dioxane. (e) HOBt, EDCI, DCM.

Scheme 3.

Reagents and conditions: (a) **4**, HOBT, EDCI, DCM. (b) (i) TFA, DCM; (ii) azido-carboxylic acids, HOBT, EDCI, DCM. (c) Sodium ascorbate, CuSO₄, H₂O/THF (1:1).

Table 1

Protective potency of bivalent compounds in MC65 cells.

Compounds	EC ₅₀ (nM)*
21MO	81.86 ± 11.91
23MO	106.68 ± 8.20
25MO	74.13 ± 15.92
27MO	140.49 ± 15.19
29MO	136.16 ± 29.52
31MO	237.85 ± 59.76
33MO	132.44 ± 56.55
35MO	359.48 ± 141.46
37MO	102.73 ± 5.60
39MO	105.28 ± 4.65

* MC65 cells were treated with bivalent compounds over a range of concentrations for 72 h and the viability was assessed by MTT assay. The resulting EC₅₀ values are given as mean ± SEM from three independent experiments.