A small bifunctional chelator that modulates $A\beta_{42}$ aggregation

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Abstract: Multifunctional compounds that can modulate amyloid-β (Aβ) aggregation and interact with metal ions hold considerable promise as therapeutic agents for Alzheimer's disease (AD). Using the copper-catalyzed azide-alkyne cycloaddition reaction, a novel bifunctional chelator 2-(1-(4-(dimethylamino)benzyl) -1H-1,2,3-triazol-4-yl)phenol (L1) was synthesized. L1 contains a bidentate metal-binding unit, and a pendant dimethylamino moiety. The product was characterized by ^{1}H NMR, ^{13}C NMR, and MS. The metal-binding properties of L1 were probed by UV-visible spectroscopy to determine Cu:L stoichiometry. L1 was determined to limit Aβ aggregation at 48 h via a ThT assay. In addition, L1 complies with Lipinski's rules and calculated logBB values for potential drug-likeness and BBB permeability. These results suggest that L1 is a suitable candidate for further study as a multifunctional compound to treat AD.

Keywords: β-Amyloid, Alzheimer's disease, click chemistry, copper ions

Introduction

With an aging population and an increase in life span, Alzheimer's disease (AD) is of major concern all over the world, and there is a lack of effective pharmacotherapy options.[1] There are many factors that have been shown to play an important role in AD, such as amyloid-β (Aβ) aggregation,[2-3] hyperphosphorylation of tau protein,[4-5] metal dyshomeostasis,[6-7] oxidative stress,[8-10] mitochondrial dysfunction and reduced levels of acetylcholine (ACh). The available therapies for AD focus mainly on cholinesterase inhibition.[1] However, the development of new therapies has shifted from single-target molecules to multi-target compounds due to the complexity and multiple etiologies of AD.[11] Many "multi-target" agents have been reported recently, such as AChE inhibitors with metal chelation properties, and AchE-induced Aβ aggregation inhibitory activity,[12] BACE1 inhibitors bearing AChE inhibitory activity,[13] BACE 1 inhibition and metal chelation,[14] and cholinesterase inhibitors with both antioxidant and neuroprotective properties.[15] Aβ aggregation and metal dysregulation are two examples of promising targets for next-generation drug therapies that are under investigation in current studies.[11] Examples of promising target molecules are shown in Fig.1.[16-21] Aβ is derived from the proteolytic cleavage of APP by β- and γ-secretases.[22] The aggregation of Aβ leads to the formation of senile plaques (SP), which is one of the hallmarks of AD brains,[2] where the plaques cause neuronal destruction and are associated with oxidative stress, mitochondrial dysfunction, loss of membrane integrity, abnormal calcium homeostasis, and induced apoptosis.[23] There is a change in metal levels in the brain of an AD patient, with high levels of metals (ca. 0.4 mM Cu; 1.0 mM Zn; 0.9 mM Fe) being found within SP from AD brain tissue compared to healthy tissue.[24-26] Metal ions have been shown to accelerate aggregation of Aβ; stabilizing neurotoxic oligomers and showing Fenton-like reactions of Aβ-bound redox active metal ions.[27] Furthermore, extensive evidence supports the connection between these two targets, probably due to the role played by trace elements during the misfolding process that occurs in Aβ aggregation.[11]

Considering the crucial roles of Aβ and metal ions in AD pathology, we synthesized a multifunctional ligand that can simultaneously impact on Aβ, metal ions and metal-Aβ species. The ligand was designed based on the direct incorporation of metal-binding atom donors into the structural framework of an Aβ-interacting compound. Click chemistry can be used effectively to connect various functional entities through a copper-catalyzed Huisgen-type 1,3-dipolar cycloaddition of terminal alkynes and organic azides. A series of dual-function triazole-pyridine ligands[16] and multifunctional quinoline-triazole ligands[17] have been previously reported by our group (**Fig.1**). Both these series were designed to have two nitrogen (N) donor atoms and different side chains that may modulate the affinity for the metal ion and/or the Aβ-peptide. The triazole-pyridine series interacts with hydrophilic N-terminal residues of Aβ and demonstrate an ability to limit metal-induced Aβ aggregation.[16] The quinoline-triazole series weakly interact with the Aβ peptide, and only modify the aggregation profile of the Aβ peptide in the presence of excess Cu ions (1.4:1).[17] In this paper, we focused on the design of a multifunctional ligand with increased peptide interactions, and a higher affinity for Cu. In structure-reactivity investigations of multifunctional ligands in both the absence and presence of metal ions for their antiamyloidogenic characteristics, it was evident that the dimethylamino group is a key component for the activity of the ligand.[28-29] Based on a structure-mechanism design strategy[18] and the previous work,[16-17, 28-29] a novel triazole-phenol derivative was designed (**Scheme 1**.) inspired by the metal chelator clioquinol (CQ) and a known Aβ imaging agent, a *p*-I-stilbene derivative.[30] The metal-binding properties of the new ligand, its interaction with Aβ, as well as its potential blood-brain barrier (BBB) permeability were evaluated.

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Fig.1. Examples of multifunctional metal-binding agents.

Scheme1. Incorporation approach to design small molecule to target metal-Aβ species and regulate their aggregation.

Experimental

Some azides are hazardous, and to avoid injury, follow safe laboratory practices, wear appropriate protective equipment and use appropriate shielding equipment. Azides can decompose violently upon heating, shock and/or friction. Only small quantities should be prepared at a single time. All common chemicals were purchased as reagent grade from Aldrich and used without further purification. All of the solvents were treated according to general methods. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AV-400 or AV-500 instruments. Mass spectra were obtained on an Agilent 6210 time-of-flight electrospray ionization mass spectrometer. UV-vis measurements were performed on a TU-1901 double-beam UV-Vis spectrophotometer. The Thioflavin T assay was measured using a Synergy 4 Fluorometer plate reader from BioTek.

Synthesis of 2-(1-(4-(dimethylamino)benzyl)-1H-1,2,3-triazol-4-yl)phenol (L1)

4-(*N,N*-dimethylamino)benzylalcohol **2** was synthesized according to a reported method.[31] ^{1}H NMR, (400MHz, $CDCl_3$): δ 7.31-7.26 (m, 1H), 6.80-6.73(m, 1H), 4.60(s, 1H), 2.98(s, 3H). 4-(chloromethyl)-*N,N*-dimethylaniline **3** and 4-(azidomethyl)-*N,N*-dimethylaniline **4** were synthesized according to previously reported procedures.[32] 4-(chloromethyl)-*N,N*-dimethylaniline: ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85–7.78 (m, 1H), 7.57–7.51 (m, 1H), 4.58 (s, 1H), 3.21 (s, 3H). 4-(azidomethyl)-*N,N*-dimethylaniline: ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26–7.22 (m, 1H), 6.79–6.75 (m, 1H), 4.27 (s, 1H), 3.01 (s, 3H). *o*-((Trimethylsilyl)ethynyl)phenol **6** was synthesized from 2-iodophenol **5** according to previously reported procedure[33] to afford the title compound as a red brown oil in 56% yield . ^{1}H NMR (400 MHz, Chloroform-*d*): δ 7.37 (dd, *J* =7.7, 1.6 Hz, 1H), 7.29–7.24 (m, 1H), 6.97 (dd, *J* = 8.3, 1.0 Hz, 1H), 6.88 (td, *J* = 7.6, 1.1 Hz, 1H), 5.86 (s, 1H), 0.31 (s, 9H); ^{13}C NMR (400 MHz, Chloroform-*d*): δ 98.92, 102.39, 109.49,114.52, 120.21, 130.66, 131.57, 157.07.

Synthesis of 2-(1-(4-(dimethylamino)benzyl)-1*H*-1,2,3-triazol-4-yl)phenol **L1**: In a 20 mL vial, *o*-((trimethylsilyl)ethynyl)phenol **6** (194.4mg, 1.02 mmol) and 4-(azidomethyl)-*N,N*-dimethylaniline **4** (184.4 mg, 1.05 mmol) were dissolved in 6 mL of methanol. In a separate 20 mL vial, $CuSO_4 \cdot 5H_2O$ (125.5 mg, 0.5 mmol), K_2CO_3 (256.3 mg, 1.85 mmol) and L-ascorbic acid (176.12 mg, 1.0 mmol) were dissolved in 6 mL of H_2O. The aqueous solution was added dropwise to the stirring methanol solution. Pyridine (0.5 mL) was added slowly to the resulting mixture and the solution was stirred at room temperature for overnight. Chelex resin was added and the solution was stirred for an additional 2 h. The solution was filtered, concentrated, and the residue was diluted with H_2O/ CH_2Cl_2 (1:1). The aqueous layer was extracted with CH_2Cl_2, combined organic layers were dried with Na_2SO_4, concentrated under reduced pressure, purified by silica gel chromatography (CH_2Cl_2/MeOH 99:1) to afford a pale yellow solid (30 mg, 10% yield). ^{1}H NMR (500 MHz, Chloroform-*d*): δ 10.95 (s, 1H), 7.69 (s, 1H), 7.34 (dd, *J* = 7.8, 1.6 Hz, 1H), 7.25 (d, *J* = 8.3 Hz, 2H), 7.23 –7.20 (m, 1H), 7.06 (dd, *J* = 8.2, 1.2 Hz, 1H),6.86 (td, *J* = 7.5, 1.2 Hz, 1H), 6.74 (d, *J* = 8.6 Hz, 2H), 5.50 (s, 2H), 3.00 (s, 6H); ^{13}C NMR (400 MHz, Chloroform-*d*): δ 40.37, 54.45, 112.56, 114.08, 117.58, 118.48, 119.35, 120.93, 125.84, 129.57, 129.67, 147.81, 150.84, 155.89; ESI(-)-MS (m/z): $[M-H]^-$ Calcd for $C_{17}H_{18}N_4O$: 293.1403; Found 293.1460.

Predictability of drug-like/BBB permeability

Using a web-based program,[34] several physicochemical properties were determined in order to predict the drug-like properties and blood-brain barrier (BBB) permeability of the 2-(1-(4-(dimethylamino)benzyl)-1*H*-1,2,3-triazol-4-yl)phenol compound. Molecular weight (MW), calculated logarithm of the octanol-water partition coefficient (clogP), H-bond acceptors (HBA), H-bond donors (HBD) and total potential surface area (TPSA) were calculated to determine the drug-like properties of the 2-(1-(4-(dimethylamino)benzyl)-1*H*-1,2,3-triazol-4-yl)phenol.[35] Clark's equation[36] (Eq. (1)) was used to determine the log BB, which is a common measure of the degree of BBB penetration.

$$\log BB = -0.0148 \times TPSA + 0.152 \times clogP + 0.139 \quad (1)$$

Metal binding studies

Metal binding studies were performed by varying the molar fractions of $CuCl_2$ from 0 to 1 (0 to 50 μM) in 10% DMSO/ HEPES Buffer pH 7.4 in the presence of ligand to obtain UV-visible spectra. An absorbance maximum was assigned as interaction of metal and ligand for each solution, which gave the determination of the metal:ligand ratio in the complex of ligand-Cu(II).

ThT Assay

$A\beta_{1-42}$ peptide was purchased from Cellmano Biotech Limited, monomerized according to a reported procedure[37]

and stored in a -80˚C freezer until use. The monomeric film was dissolved in 1:1 DMSO:ddH_2O to a final volume of 200 μL and the concentration was determined in a Thermo Nicolet UV nanodrop instrument (ε = 1450 M^{-1}). In a 96-well plate the peptide (5 μM) was incubated at 37˚C with agitation in the presence and absence of $CuCl_2$ (5 μM) and/or ligands (15 μM) in 10% DMSO in 0.1 M PBS pH 7.4. PBT2 has been shown to influence Aβ aggregation in the presence of Cu,[38-39] and was used as a control. Thioflavin T (ThT) was added to the wells at 5 μM, and fluorescence (λ_{ex} 404 nm, λ_{em} 477 nm) measured in quadruplicate at 0, 8, 24 and 48 h.

Results and Discussion

The synthesis of a new metal-binding compound towards Alzheimer's disease was performed in a few steps (**Scheme 2**). The key reaction in the synthesis of the target compound is the click chemistry between aryl azide and terminal alkyne. On the one hand, the aryl azide was obtained by reduction, chlorination, and azidation from 4-dimethylaminobenzaldehyde **1** in high yields. On the other hand, the protected alkyne was synthesized by a Sonogashira coupling reaction between 2-iodophenol **5** and trimethylsilylacetylene. The coupling between the aryl azide and the terminal alkyne was realized by a copper-catalyzed Huisigen azide-alkyne cycloaddition reaction where the deprotection of the alkyne function and the coupling were performed in the same step. The low yield could be due to the ligand binding the copper thus limiting the coupling reaction. In the future, an accelerating ligand could be added to enhance the click chemistry reaction.[40-41]

Scheme 2. Reagents and conditions: (a) $NaBH_4$, MeOH, reflux, 3 h, 98%. (b) $SOCl_2$, DCM, r.t., 12 h, 95%. (c) NaN_3, DMF, r.t., 30 h, 62%. (d) trimethylsilylacetylene, $PdCl_2(PPh_3)_2$, Et_3N, CuI, dioxane, 45℃, 5 h, 56%. (e) Pyridine, K_2CO_3, L-ascorbic acid, $CuSO_4$, CH_3OH/H_2O, r.t., 12 h, 10%.

The Lipinski rule of five[35] describes parameters that predict whether a compound will exhibit favorable pharmacokinetic properties. These parameters include MW, HBA, HBD and cLogP, and can be calculated using a web-based program[34]. The values for cLogP and TPSA can be used to calculate the log BB value using Clark's equation (Eq. (1)), which can inform potential BBB permeability. Log BB values ＞3.0 indicate readily crosses BBB and values ＜−1.0 indicate poorly distributed to the brain.[42] The predicted parameters for L1 are promising in terms of drug-likeness, however the calculated log BB value for BBB permeability is little low suggesting moderate distribution to the brain (**Table 1**). While we did not test the antioxidant properties of L1, we expect the phenol moiety to exhibit ROS quenching effects as reported for similar derivatives.[43-45]

Table 1. Values (MW, clogP, HBA, HBD, TPSA, logBB) for ligand

Calculation	MW[a]	clogP[b]	HBA[c]	HBD[d]	TPSA (Å)[e]	logBB[f]
Ligand	294.36	2.96	5	1	54.18	− 0.21
Lipinski's rules and others	≤500	≤5	≤10	≤5	≤90	> 3.0 (readily); < −1.0 (poorly)

[a]MW = molecular weight; [b]clogP = calculated logarithm of the octanol-water partition coefficient; [c]HBA = H-bond acceptors; [d]HBD = H-bond donors; [e]TPSA = total potential surface area; [f]logBB= −0.0148TPSA + 0.152 clogP + 0.130.

The interaction of the ligand with Cu^{2+} was probed using UV-vis spectroscopy. Upon addition of $CuCl_2$ to a solution of ligand, decreased intensity and shifts of the optical band at *ca.* 250 nm and 290 nm were observed relative to ligand. A new band at *ca.* 320 nm was observed at the same time (**Fig. 2A**). Changes in the intensity of the ligand-based transition, along with the observation of new absorption indicated Cu^{2+} binding to ligand. To determine the ligand-Cu(II) stoichiometry of the complex, the technique of continuous variation (also called Job's method) was used by preparing solutions of ligand and $CuCl_2$ so that the sum of concentrations of both species was constant in all samples, but the proportions of both components varied between 0% and 100%. The UV spectra were recorded and the absorbance at 320 nm, which corresponds to a ligand to metal charge transfer absorption band, was plotted versus the mole fraction of $CuCl_2$. The concentration of complex (ligand-Cu(II)) increases as the concentration of $CuCl_2$ is increased, until it reaches a maximum which corresponds to the optimal metal:ligand ratio of the complex. As shown in **Fig.2B**, the absorption at 320 nm reaches a maximum over a range of % Cu values. The broadness of the peak suggests that a mixture of 1:1 and 1:2 metal:ligand species are present in solution at pH 7.4.[46-47]

Fig. 2. (A) UV-vis spectra of ligand and copper chloride at different mole fraction of $CuCl_2$ (C_{tot} = 50 μM) in HEPES buffer (0.1M, PH=7.4). (B) Determination of the stoichiometry of complex ligand-Cu(II) by Job's method.

ThT shows an increase in fluorescence in the presence of $A\beta_{1-42}$ fibrils[48] and was used to investigate the influence of L1 on the aggregation pattern in the presence and absence of Cu^{2+}. PBT2 (Fig.1) was used for comparison with the

ligand synthesized in this work, due to the promising characteristics of this ligand in AD models (**Fig.3**). Over time, $A\beta_{1-42}$ shows an increase in ThT fluorescence, reaching its maximum fluorescence at 48 h. Up to 24 h of incubation both L1 and PBT2 not show any difference in aggregation pattern, however at 48 h L1 shows a significant decrease in ThT fluorescence intensity, suggesting that the ligand interacts with the peptide to limit the formation of higher MW fibrils. In the presence of Cu^{2+}, there is no increase in ThT fluorescence, in accordance with results shown previously that copper stabilizes low molecular weight species.[49-50] L1 does not appear to prevent Cu^{2+} interaction with $A\beta_{1-42}$, suggesting that the Cu-affinity of this derivative is lower than that of the Aβ peptide. While PBT2 does not change the aggregation pattern of $A\beta_{1-42}$ under Cu-free conditions, in the presence of Cu^{2+} a small increase in ThT fluorescence is obverted, thus showing that PBT2 can influence the Cu-Aβ interaction.

Fig. 3. ThT fluorescence of (A) $A\beta_{1-42}$ in the presence and absence of ligands; (B) $A\beta_{1-42}$ with $CuCl_2$ in the presence and absence of ligands.

Conclusion

In summary, using a click reaction between *o*-((trimethylsilyl)ethynyl)phenol and 4-(azidomethyl)-*N,N*-dimethylaniline, a novel triazole-phenol derivative L1 was synthesized. This triazole-phenol derivative conformed to Lipinski's rules and the calculated logBB value for potential blood-brain barrier (BBB) permeability. Metal binding properties and Aβ interaction properties of this triazole-phenol derivative were studied by UV-vis and a ThT Assay. The further synthesis and functional studies of similar compounds are underway in our group.

Supplementary data

Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/.

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Figures and Schemes:

Fig.1. Examples of multifunctional metal-binding agents.

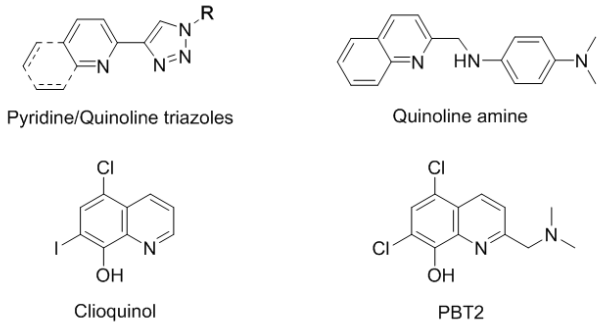


Fig. 2. (A) UV-vis spectra of ligand and copper chloride at different mole fraction of $CuCl_2$ (C_{tot} = 50 μM) in HEPES buffer (0.1M, PH=7.4). (B) Determination of the stoichiometry of complex ligand-Cu(II) by Job's method.

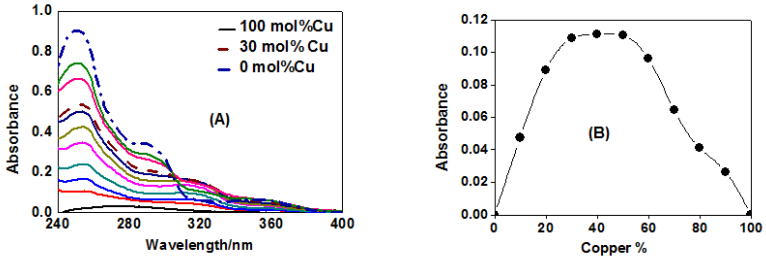
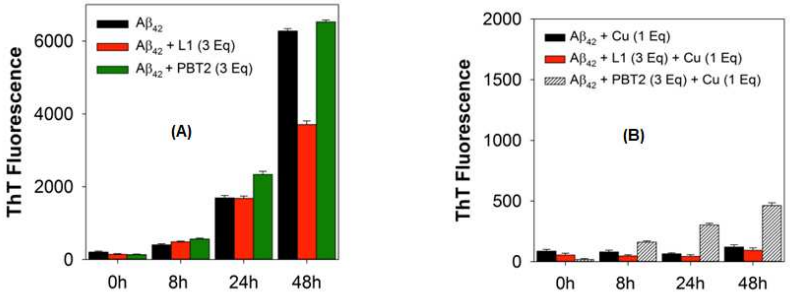
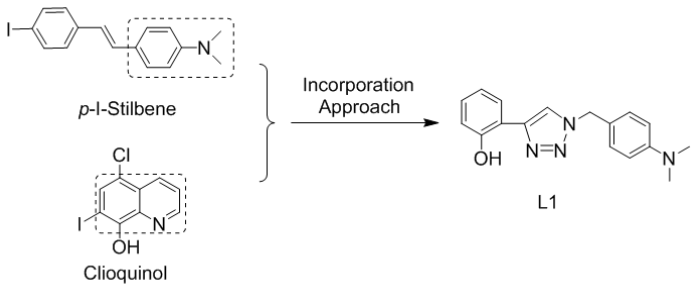


Fig. 3. ThT fluorescence of (A) $A\beta_{1-42}$ in the presence and absence of ligands; (B) $A\beta_{1-42}$ with $CuCl_2$ in the presence and absence of ligands.



Scheme1. Incorporation approach to design small molecule to target metal-Aβ species and regulate their aggregation.



Scheme 2. Reagents and conditions: (a) $NaBH_4$, MeOH, reflux, 3 h, 98%. (b) $SOCl_2$, DCM, r.t., 12 h, 95%. (c) NaN_3, DMF, r.t., 30 h, 62%. (d) trimethylsilylacetylene, $PdCl_2(PPh_3)_2$, Et_3N, CuI, dioxane, 45℃, 5 h, 56%. (e) Pyridine, K_2CO_3, L-ascorbic acid, $CuSO_4$, CH_3OH/H_2O, r.t., 12 h, 10%.