α-Conotoxin Dendrimers Have Enhanced Potency and Selectivity for Homomeric Nicotinic Acetylcholine Receptors

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α-Conotoxin Dendrimers Have Enhanced Potency and Selectivity for Homomeric Nicotinic Acetylcholine Receptors

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Supporting Information

ABSTRACT: Covalently attached peptide dendrimers can enhance binding affinity and functional activity. Homogenous di- and tetravalent dendrimers incorporating the α7-nicotinic receptor blocker α-conotoxin ImI (α-ImI) with polyethylene glycol spacers were designed and synthesized via a copper-catalyzed azide−alkyne cycloaddition of azide-modified α-ImI to an alkyne-modified polylysine dendron. NMR and CD structural analysis confirmed that each α-ImI moiety in the dendrimers had the same 3D structure as native α-ImI. The binding of the α-ImI dendrimers to binding protein Ac-AChBP was measured by surface plasmon resonance and revealed enhanced affinity. Quantitative electrophysiology showed that α-ImI dendrimers had ∼100-fold enhanced potency at hα7 nAChRs (IC_{50} = 4 nM) compared to native α-ImI (IC_{50} = 440 nM). In contrast, no significant potency enhancement was observed at heteromeric hα3β2 and hα9α10 nAChRs. These findings indicate that multimeric ligands can significantly enhance conotoxin potency and selectivity at homomeric nicotinic ion channels.

Multimeric ligands attached to a single molecular scaffold may have more favorable binding affinity than the corresponding monomeric ligands.[1] Further, multimeric ligand dendrimers with highly branched architecture can provide fine control over the size and structure of the constructs.[2] Though a wide selection of dendrons are available, polylysine dendrimers assembled from L-lysine amino acids are of particular interest owing to their synthetic expedience and low toxicity.[3,4] A wide range of ligand types, including truncated antibodies[5] and carbohydrate analogues,[6] have been conjugated to dendrimeric scaffolds via diverse chemical ligation chemistries. However, the conjugation of multiple peptide ligands to dendrimers has proven more challenging, especially for highly structured peptides.[7,8] Accordingly, a greater focus has been on linear peptides,[9] with only a few studies of well-structured peptide dendrimers being reported.[8,10]

In a strategy to enhance the potency of α-conotoxin ImI (α-ImI) at the homomeric neuronal α7 nicotinic acetylcholine receptor,[11] we designed and synthesized a range of α-ImI dendrimers. Whereas the development of α-ImI analogues with improved antagonistic activity has attracted considerable attention,[12] to date no multimeric α-ImI constructs have been investigated. Given the existence of a multivalency effect in multimeric GPCR ligands,[13] we anticipated that α-ImI dendrimers could exhibit enhanced binding affinity and potency. Our initial focus was on dimeric and tetrameric dendrimers of α-ImI.

Our synthetic approach utilized the copper-catalyzed azide−alkyne cycloaddition (CuAAc) reaction to attach the azido component of α-ImI to alkyne-polylysine dendrons (Scheme 1). The highly efficient CuAAc reaction is particularly suitable for the immobilization of peptides containing multiple functional groups, as the azide and alkyne reaction partners are inert to other functional groups under typical reaction conditions.[14,15] We initiated the synthesis of azide-modified α-ImI **1** and alkyne lysine dendrons **2** and **3** using solid-phase Fmoc chemistry.

The azido α-ImI peptide **1** (denoted as azido-PEG(9)-ImI) was folded regioselectively, and the N-terminal azide functionality was introduced via azido-PEG(9) acid (Supporting Information, Scheme S1). PEG addition not only enhanced the peptide construct solubility[16] but also provided a spacer segment to vary flexibility, length, and accessibility of the α-ImI ligands. To enable CuAAc reaction with **1**, a similar methodology was used to prepare di- and tetra-polylysine dendrons with peripheral propargyl groups (Scheme S2).[17] The dimer (**2**) and tetramer (**3**) alkyne lysine dendrons, together with azide **1**, were purified and characterized by LC-MS (Figures S1 and S2, Table S1).

The chemoselective CuAAc reaction was then undertaken in the presence of tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]-amine together with the ligand $CuSO_4$ and sodium ascorbate in DMF/H_2O (9:1 v/v) overnight at room temperature (Scheme S3). The reactions were monitored by RP-HPLC to display new peaks corresponding to the desired di- and tetravalent peptide dendrimers **4** and **5** (denoted as 2xImI-PEG(9)-D and 4xImI-PEG(9)-D). HPLC findings were supported by ESI-MS data that indicated no incomplete

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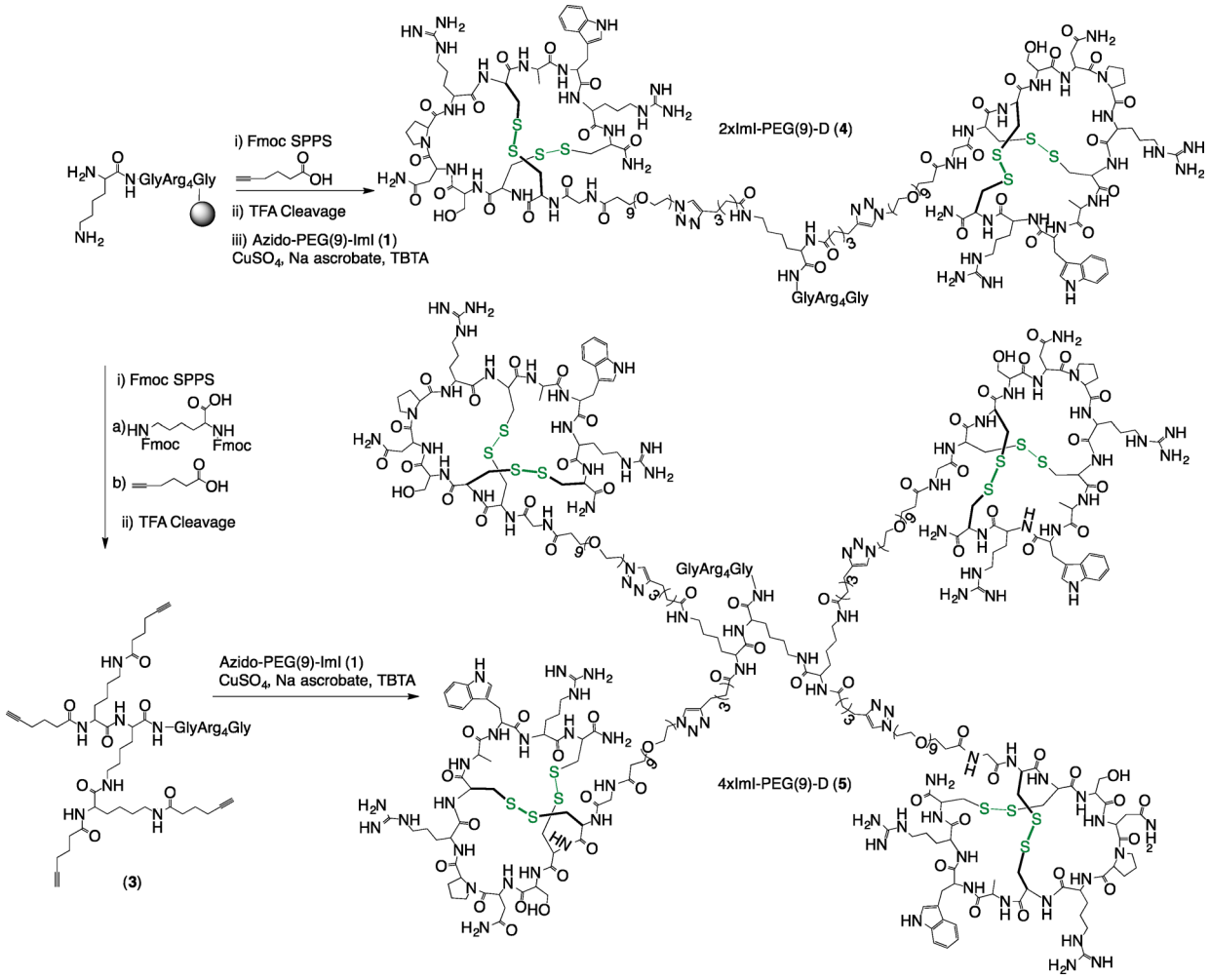
Scheme 1. Synthesis of Divalent (4) and Tetravalent (5) α-ImI Dendrimers[a]



[a]Native α-ImI sequence: GCCSDPRCAWRC [1-3,2-4 disulfide bonds].

peptide dendrimers were present (Figures S3 and S4, Table S1). No reduction of azido peptide **1** containing disulfide bonds was observed (Figure S5); therefore, excess azido-peptide **1** could be recovered and reused in further reactions. The reaction mixtures were purified by semipreparative RP-HPLC to give homogeneous (>90% purity) preparations of dendrimers **4** and **5**.

CD spectroscopy was used to study the conformation of the α-ImI moieties in dendrimers **4** and **5**. As shown in Figure 1a, the dendrimeric α-ImI compounds **4** and **5**, together with azide **1** and α-ImI, had similar secondary structures with two minima at 207 and 222 nm, suggesting the existence of characteristic helical elements. In contrast, the tetravalent dendron core showed random coil structure (Figure S6), indicating that the helical elements in compounds **4** and **5** arose from the attached α-ImI. 2D NMR at 900 MHz was then used to examine the three-dimensional structures of dendrimeric α-ImI's **4** and **5**. Although the individually attached α-ImI are in different chemical environments, due to the asymmetrical dendron structure, the NMR spectra of the α-ImI dendrimers **4** and **5** revealed a single set of resonances for the α-ImI moieties. This indicates that each α-ImI unit in the dendrimers is chemically equivalent. Furthermore, only minor changes in αH chemical shift between free and tethered α-ImI's **4** and **5** were observed. These findings indicate that α-ImI freely rotates with negligible

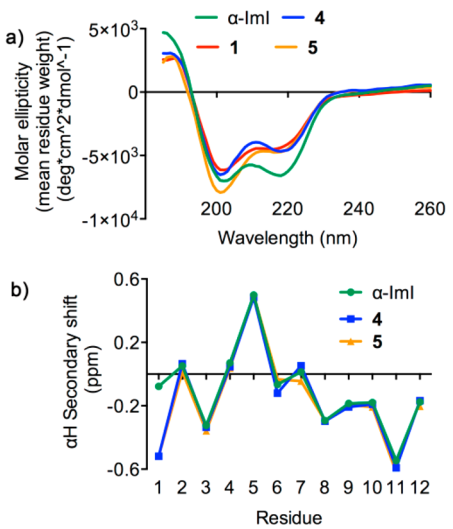


Figure 1. (a) CD spectra of α-ImI, azide **1**, 2xImI-PEG(9)-D (**4**), and 4xImI-PEG(9)-D (**5**). (b) Secondary chemical shifts of α-ImI, **4**, and **5**. The secondary chemical shifts were determined by subtracting random coil shift[18] from the αH shift.

structural perturbation in the dendrimeric form (Figure 1b, Tables S2–S4). Together, the data suggest that each α-ImI in

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the dendrimer is structurally intact and free to interact with nAChRs independently, thus allowing potential multivalent interactions.

The binding interactions between the α-ImI dendrimers **4** and **5** and Ac-AChBP were studied using a surface plasmon resonance (SPR) biosensor instrument. Ac-AChBP is a homologue of the nAChR ligand-binding domains with pharmacological properties similar to those of α7 nAChRs.[19] As shown in Table 1, α-ImI dendrimers **4** and **5** bound to Ac-AChBP more tightly than monovalent α-ImI (Figures S7 and S8) with 5−10-fold lower K_D values, indicative of multivalent enhancement. The improved affinity of the 2-mer **4** resulted from faster on-rates and slower off-rates, while for the 4-mer **5** only the on-rate was altered. The enhanced binding affinity in dendrimeric ImI can be attributed to the concomitant binding to two binding sites on the homomeric Ac-AChBP. To achieve binding at two sites according to the model of the Ac-AChBP (Figure S9a), the linker would need to span a minimum distance of 62.5 Å. As the maximum linker length between two ImI moieties in dendrimeric α-ImI **4** is ∼103 Å, this linker can accommodate two-site binding.

Table 1. Kinetic Binding Constants of Monomer and α-ImI Dendrimers at Ac-AChBP, Determined from SPR Experiments[a]

	k_{on} ($\times 10^2$ Ms^{-1})	k_{off} ($\times 10^{-3}$ s^{-1})	K_D (μM)	IC_{50} (nM)
α-ImI	1.1 ± 0.4	4 ± 1	45.4 ± 28.8	440 ± 30
1	1.5 ± 0.2	4 ± 1	24.1 ± 8.5	517 ± 70
4	3.7 ± 0.3	2 ± 0.1	5.2 ± 0.6	4.3 ± 0.9
5	4.6 ± 0.1	4 ± 1	9.4 ± 3.9	6.7 ± 0.8

[a] IC_{50} values were determined on human α7 nAChRs expressed in *Xenopus* oocytes. All values are means ± SEM of three separate experiments, each performed in triplicate.

The functional activities of the α-ImI azide **1** and the α-ImI dendrimers **4** and **5** were further examined directly on human α7, α9α10, and α3β2 nAChR subtypes expressed in *Xenopus* oocytes. Wild-type α-ImI monomer reversibly inhibited ACh (100 μM) currents mediated by hα7 nAChRs in a concentration-dependent manner and with an IC_{50} of 440 nM (n = 4). This value is similar to that reported previously (595 nM).[20] At the hα7 receptor, azide **1** and the dendrimeric α-ImI **4** and **5** reversibly inhibited ACh-evoked amplitude in a concentration-dependent manner with IC_{50} values of 517 ± 70, 4.3 ± 0.9, and 6.7 ± 0.8 nM, respectively (n = 3) (Figure 2a). These findings confirm the significant multivalency effect to dendrimeric α-ImI potency. Consistent with binding data, dimer **4** was slightly more potent than tetramer **5**, indicating increased α-ImI concentration provided no additional advantage. A similar trend in activity was observed in a functional Ca^{2+} mobilization assay using the human neuroblastoma cell line SH-SY5Y that endogenously expresses α7 nAChR, in which dendrimeric α-ImI **4** showed a ∼4-fold increase (IC_{50} = 1.3 μM) and dendrimeric α-ImI **5** displayed a ∼2-fold increase (IC_{50} = 2.8 μM) (Figure S10, Table S5).

Given that α-ImI also potently inhibits heteromeric hα3β2 (40.8 nM)[20] and weakly inhibits hα9α10 nAChRs (≥1 μM, see Figure 2b), we evaluated the relative activity of azide **1** and the dendrimeric α-ImI's **4** and **5** at these nAChR subtypes. α-ImI (1 μM) inhibited hα3β2 and hα9α10 by 99.7 ± 0.01% (n = 3) and 59.7 ± 0.06% (n = 5), respectively. Interestingly, inhibition of hα3β2 and hα9α10 nAChRs by dendrimeric α-ImI's **4** and **5**

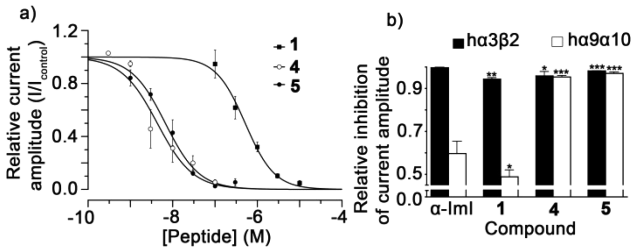

Figure 2. Inhibition of hα7, hα9α10, and hα3β2 nAChR subtypes by WT α-ImI and ImI dendrimers. (a) Concentration−response curves of inhibition of ACh-evoked current amplitude mediated by hα7 nAChR by azide **1**, 2xImI-PEG(9)-D (**4**), and 4xImI-PEG(9)-D (**5**). (b) Bar graph of relative inhibition of ACh-evoked current amplitude mediated by hα9α10 and hα3β2 receptors in the presence of 1 μM α-ImI, **1**, **4**, and **5**. Data represented as mean ± SEM, n = 3−5 (unpaired Student t test vs α-ImI; *p < 0.05, **p = 0.0001, ***p < 0.0001 vs α-ImI).

was comparable to or slightly greater than that of α-ImI, with no multivalent enhancement observed (Figure 2b). To explain the absence of any multivalency effect at heteromeric receptors where orthosteric binding sites are nonadjacent, we additionally evaluated the minimum binding distance between two nonadjacent sites, as shown in Figure S9b. The minimum distance to span two nonadjacent sites was ∼101.4 Å, similar to the maximum length of the linker in dendrimeric α-ImI **4** (103 Å). However, while both sites are theoretically accessible based on our distance calculations, nonadjacent sites are expected to be energetically less favored than adjacent sites only found in homomeric α7 nAChR. The improvement in potency and specificity makes dendrimeric α-ImI's **4** and **5** useful as highly selective probes for hα7 nAChRs.

In conclusion, we have performed efficient and versatile syntheses of α-ImI peptide dendrimers via the CuAAc ("click") reaction. These dendrimers maintain their tertiary peptide structure and are free to interact with hα7 nAChRs. In comparison to monomeric α-ImI, dendrimeric α-ImI shows significantly enhanced selectivity and functional activity at homomeric hα7 over heteromeric hα3β2 and hα9α10 nAChRs. These multivalent peptide constructs represent a promising approach to enhancing potency and selectivity of ligands for homomeric ion channels.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details of chemical procedures, NMR and CD structural detection, binding distance calculation, SPR analysis, and functional assay. 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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