SUPPORTING INFORMATION

α-Conotoxin dendrimers have enhanced potency and selectivity for homomeric nicotinic acetylcholine receptors

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Table of contents

Abbreviations

Natural occurring amino acids are abbreviated to standard single or standard three letter codes; HBTU, N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate); TFA, trifluoroacetic acid; DIEA, N,N'-diisopropylethylamine; DMF, N,N'-dimethylformamide; DCM, dichloromethane; ACN, acetonitrile; HATU, O-(7-Azabenzotriazol-1-yl)-N,N,N′ ,N′ -tetramethyluronium hexafluorophosphate; TBTA, Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine; Mtt, methyltrityl; Acm, acetamidomethyl; DMSO, dimethyl sulfoxide; TIPS, triisopropylsilane; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; NHS, N-hydroxysuccinimide; DMEM, dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; RP-HPLC, reversed-phase high-performance liquid chromatography; LC-MS, liquid chromatography coupled mass spectrometry; CD, circular dichroism, NMR, nuclear magnetic resonance; NOESY, nuclear overhauser enhancement spectroscopy; HSQC, Heteronuclear single quantum coherence spectroscopy; TOCSY, total correlated spectroscopy; AChBP, acetylcholine binding protein; nAChR, nicotinic acetylcholine receptor; FLIPR, fluorescence imaging plate reader; SPR, surface plasmon resonance.

Materials and Methods

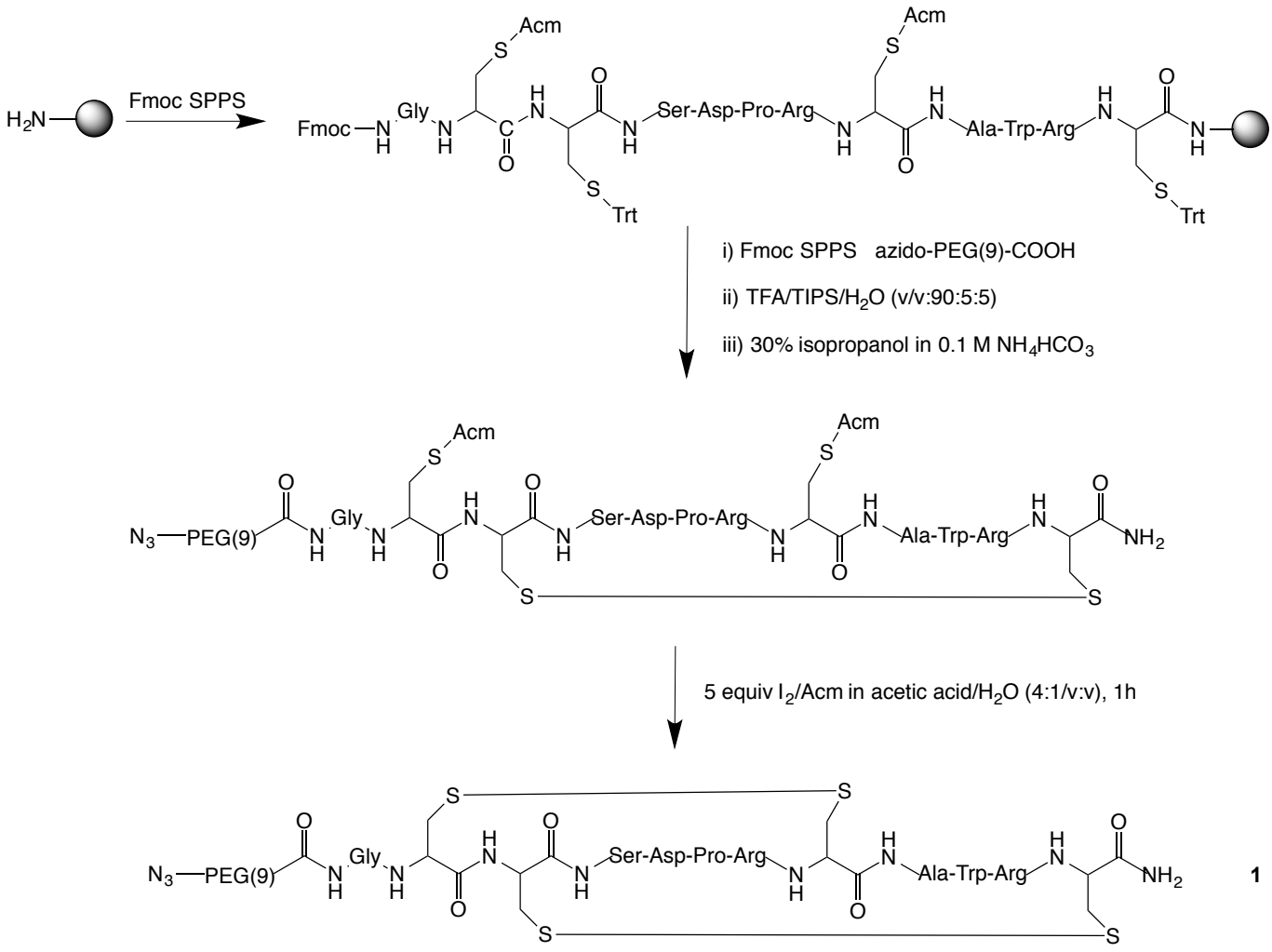
Water measuring 18.2 MΩ was used for all buffers for liquid chromatography. Screw-cap glass peptide synthesis reaction vessels with sintered glass filter frits were used throughout the synthesis. Analytical RP-HPLC was performed on a Shimadzu LC-20AD solvent delivery system equipped with a SIL-20A auto-injector and a SPD-20A UV/Vis detector using a Zorbax 300SB-C18 analytical column (300Å, 5 μm, 150 mm x 4.6 mm). Data was recorded and processed with Shimadzu Class VP software. The eluents were monitored at 214 nm and 254 nm, respectively. The HPLC solvent was made as follows: solvent A was 0.05 % TFA in H_2O, while solvent B was 0.043% TFA in ACN/H_2O (v/v: 90/10). For peptide purification, two types of columns were applied including Vydac C_{18} column (300Å, 10 μm, 250 mm x 22 mm) and Zorbax C_{18} column (300 Å, 10 μm, 250mm×10 mm). The purification was carried out by preparative RP-

HPLC using a Shimadzu LC-8A solvent delivery system. The absorbance was monitored at 214 nm and 230 nm with a Shimadzu SPD-10AV UV/Vis detector. A PE-SCIEX QSTAR Pulsar mass spectrometer was coupled with HPLC to perform mass analysis. Data acquisition and processing were carried out using Analyst software v 1.5. Circular Dichroism (CD) was performed using a Jasco JX710 spectropolarimeter.

Synthesis and oxidation of α-ImI analogue 1

N_3-PEG(9)-CO-NH-Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys-NH_2 **1**

(Cysteine connectivity: 1-3, 2-4)



Scheme S1. Synthetic route for the preparation of azido peptides **1**. The linear, reduced ImI peptide with azido-PEG(9) chain is synthesized via Fmoc SPPS. After TFA cleavage, the two disulfide bonds were formed through regioselective oxidation.

Reduced azido α-ImI **1** was assembled by Fmoc/tBu-based manual SPPS on a Rink amide resin (loading 0.71 mmol/g) using HBTU/DIEA via an *in situ* neutralization protocol[1] to activate the standard residues, and treatment with 30% piperidine/DMF (2×1min) for Fmoc deproctection. Couplings were carried out with 4 equiv of Fmoc- amino acid, 4 equiv of HBTU, and 4 equiv of DIEA in DMF for a minimum of 10 min and the acylation efficiency was monitored by the quantitative ninhydrin test.[2] For the proline residue, the efficiency was tested by the Isatin test.[3] To achieve regioselective oxidation, Fmoc-Cys(Acm)-OH were coupled in position Cys^2 and Cys^8 while Fmoc-Cys(Trt)-OH were used in position Cys^3 and Cys^{12}. 2 equiv of N_3-$(PEG)_9$-COOH activated by HATU were subsequently coupled to the N-terminal glycine residue for overnight reaction (scheme S1). After TFA cleavage (TFA:TIPS: H_2O, 90:5:5, 3 h), the mixture was filtered to remove the resin. The filtrate was transferred into cold peroxide free ether to precipitate the peptide. The supernatant was removed after centrifugation and the precipitate was dissolved in 50% ACN/ H_2O /0.0425%TFA then lyophilized. The crude reduced peptides were dissolved in ammonium bicarbonate (0.1M) buffer at pH 8 to form the first disulfide bond between Cys^3 and Cys^{12}. The oxidation products were subjected to preparative RP-HPLC system for purification. The purified peptides were then treated with iodine (1mM) to remove Acm protection and form the disulfide bond between Cys^2 and Cys^8 simultaneously. After 1 h, 0.1M ascorbic acid was added to system to quench the reaction. The fully oxidized products were purified with a preparative C_{18} column with 1 %/min linear gradient of 20-60 % solvent B at a flow rate of 15 mL/min. LC-ESI result of the purified azide peptides **1** is shown in figure S1, LC method: 1%/min linear gradient of 20-40 % solvent B at a flow rate of 1 mL/min.

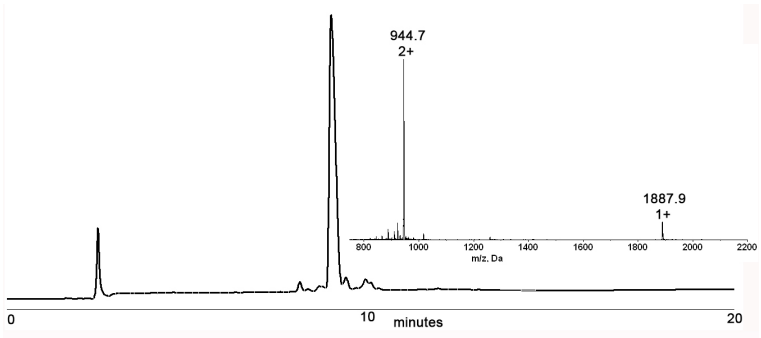
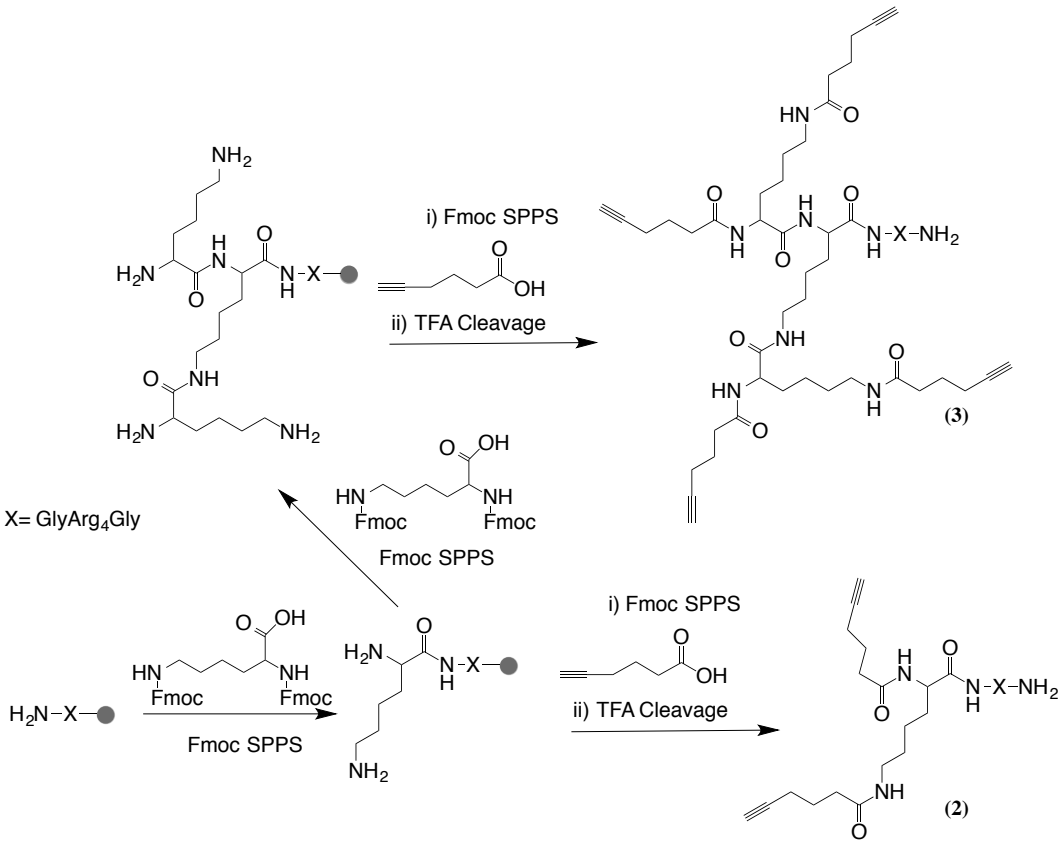


Figure S1. LC-ESI MS result of azido-PEG(9)-ImI **1.**

Synthesis of alkyne-dendrons 2&3

$GlyArg_4Gly\text{-}Lys[CO\text{-}(CH_2)_3\text{-}C{\equiv}CH]_2$ **2,** $GlyArg_4Gly\text{-}Lys[Lys]_2[CO\text{-}(CH_2)_3\text{-}C{\equiv}CH]_4$ **3**



Scheme S2. Synthetic routes for the preparation of alkyne polylysine dendrons **2&3**.

The lysine dendrons were synthesized via Fmoc-SPPS as described above (scheme S2). Specifically, a chain with a sequence of $GlyArg_4Gly$ was first coupled to the Rind Amide resin in order to decrease the loading amount (from 0.7 mmol/g to 0.29 mmol/g) and increase the hydrophilicity of the final peptide construct. Then, 4 equiv of excess bis-Fmoc-lysine per amine group were added to the coupling system to form amino-terminated dendron with 2 amino groups on outer surface, and by repeating the addition of the lysine layer, tetravalent dendron with 4 amino groups was subsequently obtained. After that, 2 equiv of excess 5-hexynoic acid per dendron-branch, activated by 0.5 M HATU, was coupled for 24 h, yielding the alkyne-dendrons **2**&**3**. Cleavage of the dendrons from resin was performed under standard conditions (TFA:TIPS:water, 90:5:5, RT, 3 h). The crude products **2**&**3** were purified with a preparative C_{18} column with 1 %/min linear gradient of 10-60 % solvent B at a flow rate of 15 mL/min. LC-ESI results of the purified alkyne dendrons **2**&**3** are shown in figure S2.

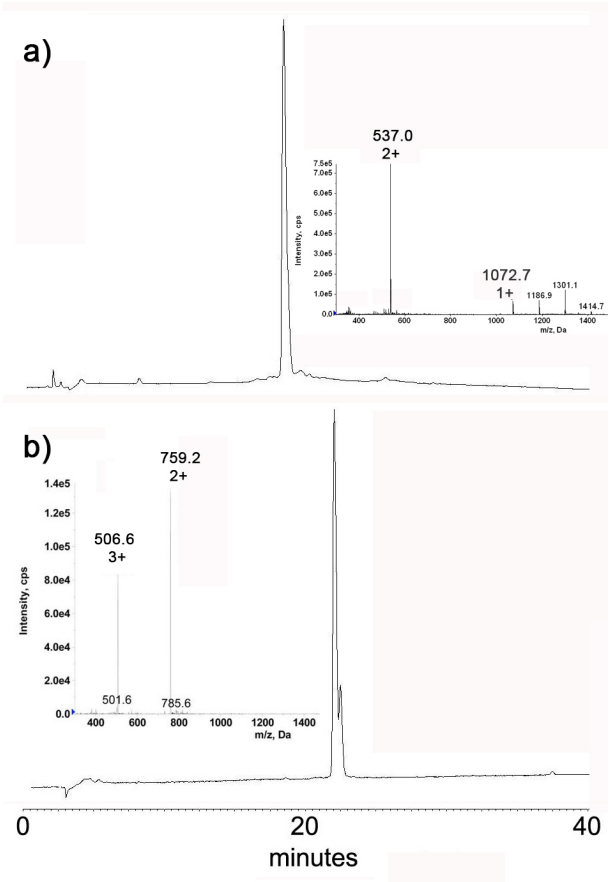
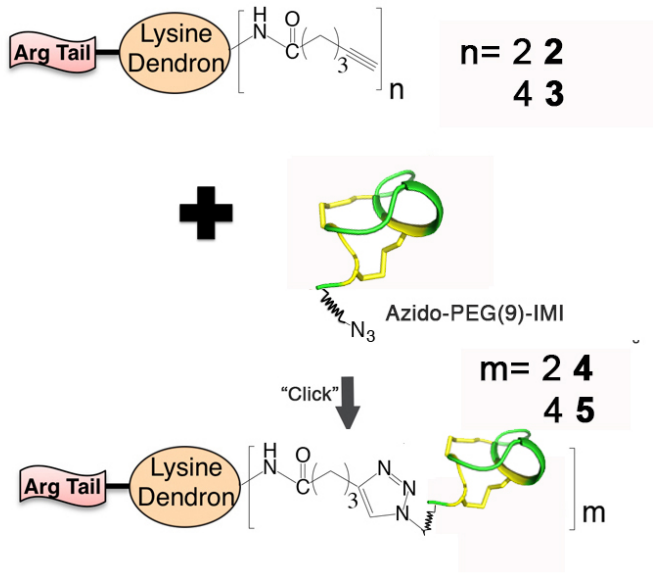


Figure S2. LC-MS results of a) 2-mer alkyne dendron **1** and b) 4-mer alkyne dendron **2**, LC method: 1%/min linear gradient of 0-40 % solvent B at a flow rate of 1 mL/min.

In-solution click reaction of alkyne-dendrons and azido-PEG(9)-ImI

$GlyArg_4Gly$-Lys[CO-$(CH_2)_3$-1,2,3-triazole-PEG(9)-CO-NH-Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys-NH_2]$_2$ **4** (Cysteine connectivity: 1-3, 2-4)

$GlyArg_4Gly$-Lys[Lys]$_2$[CO-$(CH_2)_3$-1,2,3-triazole-PEG(9)-CO-NH-Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys-NH_2]$_4$ **5** (Cysteine connectivity: 1-3, 2-4)



Scheme S3. Cu-catalyzed click ligation of azido ImI **1** and alkyne dendrimers **2&3** resulting in final ImI dendrimers **4&5** (disulfide bonds highlighted in yellow)

Alkyne-dendrons **2&3**, azido-PEG(9)-ImI **1** (2 equiv/branch), a solution of $CuSO_4$ (0.1 M, 0.2 equiv/branch) and TBTA ligand (0.2 equiv/branch) were dissolved in DMF with a dendron concentration of 5 mM. The mixture was degassed with nitrogen for 15 min and the reaction vessel was sealed with a syringe-rubber seal. A solution of sodium ascorbate (0.1 M, 0.5 equiv/branch) was added to the sealed reaction vessel by syringe

and the mixture was stirred overnight at room temperature. The reactions were monitored by analytical HPLC as shown in figures S3&S4. The reaction products were purified with a Zorbax semi-preparative C_{18} column with 0.5 %/min linear gradient of 15-60 % buffer B at a flow rate of 7 mL/min.

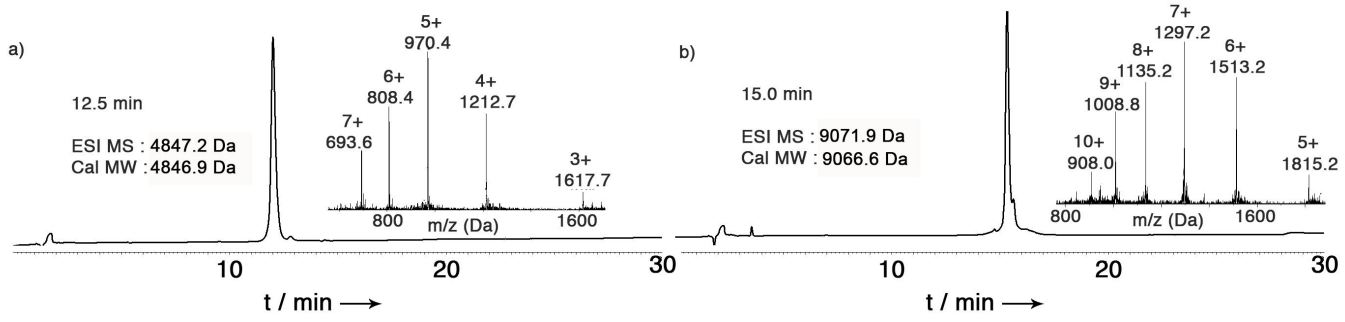


Figure S3. LC-ESI MS results of 2xImI-PEG(9)-D **4** and 4xImI-PEG(9)-D **5**, LC method is: 1%/min linear gradient of 15-45 % solvent B at a flow rate of 1 mL/min

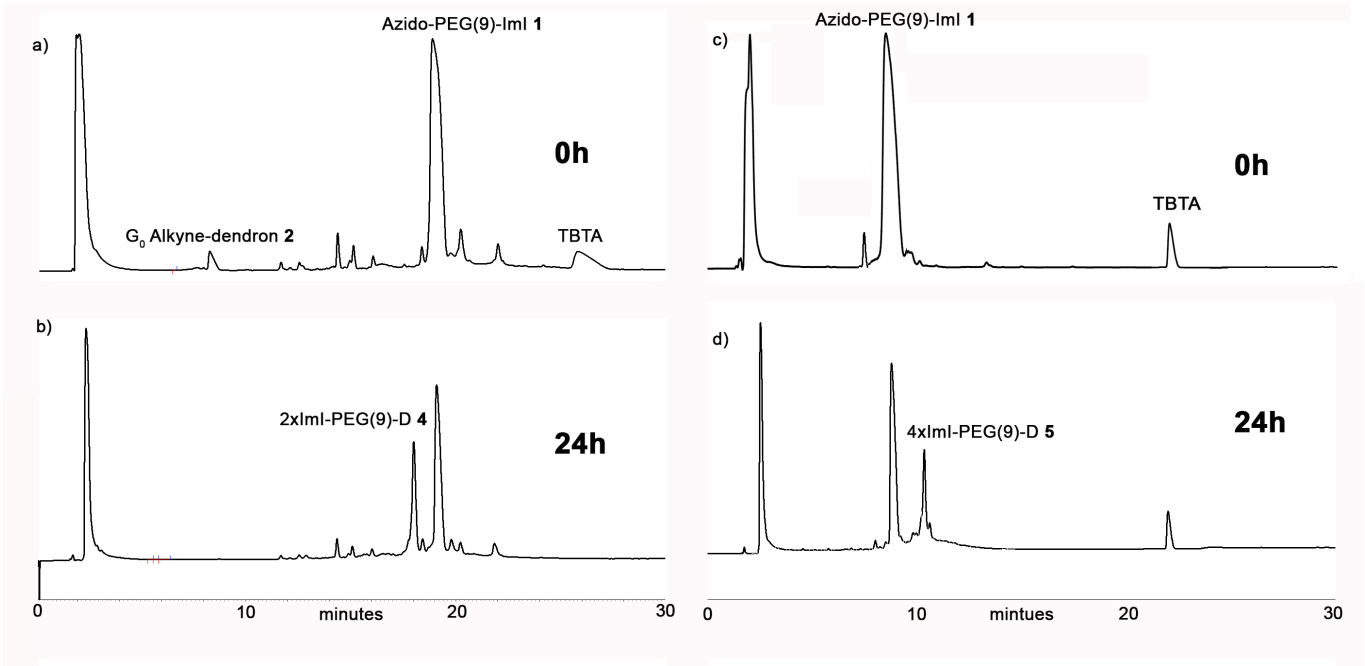


Figure S4. HPLC analysis of click reaction between azido-PEG(9)-ImI **1** and 2-mer alkyne dendron **2** after a) 0 h and b) 24 h, LC method is: 1%/min linear gradient of 10-40 % solvent B at a flow rate of 1 mL/min;

HPLC analysis of click reaction between azido-PEG(9)-ImI **1** and 4-mer alkyne dendron **3** after c) 0 h and d) 24 h, LC method is: 1%/min linear gradient of 20-50 % solvent B at a flow rate of 1 mL/min.

Stability test of azido modified ImI containing disulfide bond under click conditions

Azido-PEG(9)-ImI **1** (7 mg), a solution of $CuSO_4$ (0.1 M, 4 µL) and TBTA ligand (0.2 mg) were dissolved in 200 µL DMF. The mixture was degassed with nitrogen for 15 min and the reaction vessel was sealed with a syringe-rubber seal. A solution of sodium ascorbate (0.1 M, 10 µL) was added to the sealed reaction vessel by syringe and the mixture was stirred at room temperature. The reaction was monitored by analytical HPLC at time points of 0 min, 4h and 24h. At each time point, 10 µL mixture was taken and diluted with 90 µL solvent (0.05% TFA in ACN/H_2O (v/v: 45/55)) to run the HPLC.

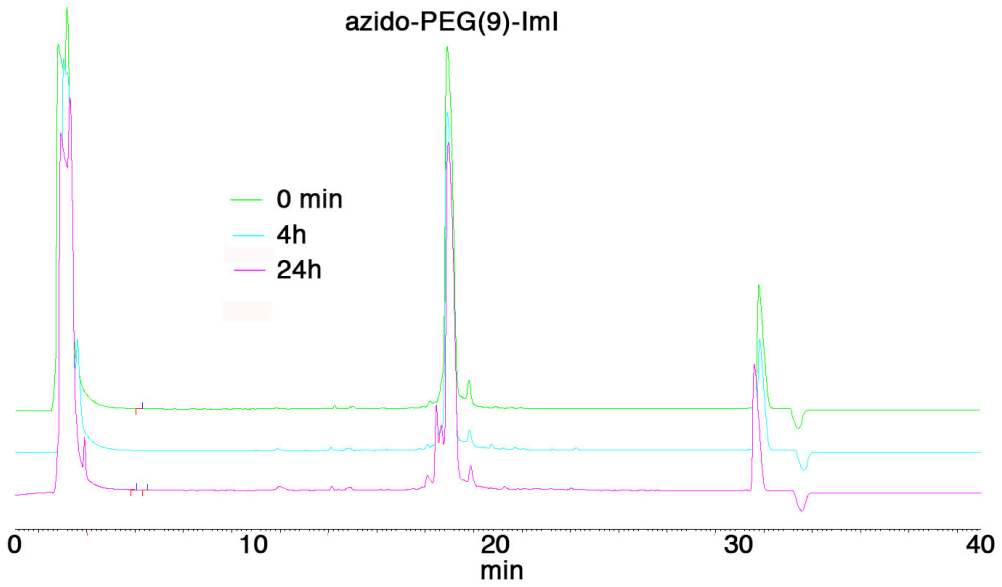


Figure S5. HPLC traces of azido-PEG(9)-ImI **1** under click conditions at 0 min, 4h and 24h, no disulfide reduction of azido ImI was observed after 24h incubation. LC method is: 1%/min linear gradient of 10-40 % solvent B at a flow rate of 1 mL/min.

Table S1. Theoretical mass and found molecular mass of the synthetic compounds **1-5**

Entry	Compound	Theoretical Mass (Da)	Found Molecular Mass (Da)
1	N_3-PEG(9)-CO-NH-Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys-NH_2 (Cysteine connectivity: 1-3, 2-4)	1887.5	1886.9
2	$GlyArg_4Gly$-Lys[CO-$(CH_2)_3$-C≡CH]$_2$	1071.9	1071.7
3	$GlyArg_4Gly$-Lys[Lys]$_2$[CO-$(CH_2)_3$-C≡CH]$_4$	1516.6	1516.4
4	$GlyArg_4Gly$-Lys[CO-$(CH_2)_3$-1,2,3-triazole-PEG(9)-CO-NH-Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys-NH_2]$_2$ (Cysteine connectivity: 1-3, 2-4)	4846.9	4847.2
5	$GlyArg_4Gly$-Lys[Lys]$_2$[CO-$(CH_2)_3$-1,2,3-triazole-PEG(9)-CO-NH-Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys-NH2]$_4$ (Cysteine connectivity: 1-3, 2-4)	9066.6	9071.9.

Circular Dichroism analysis

Circular Dichroism spectra were measured for the compounds (**1**, **3**, **4** and **5**) and α-ImI in H_2O using a Jasco JX710 spectropolarimeter. The concentrations of azide **1** and alkyne dendron **3** and wild type α-ImI are all 0.2 mg/mL, while the ImI dendrimers **4** and **5** were measured at 0.05 mg/mL. A 0.01cm path length demountable cell was used for all measurements. Data was recorded at room temperature (21) from 260-180 at 10nm/min, with resolution of 1 nm and a response time of 4s. Nitrogen flow was maintained at 10 mL/min for the duration of the measurements. Data was accumulated over three scans.

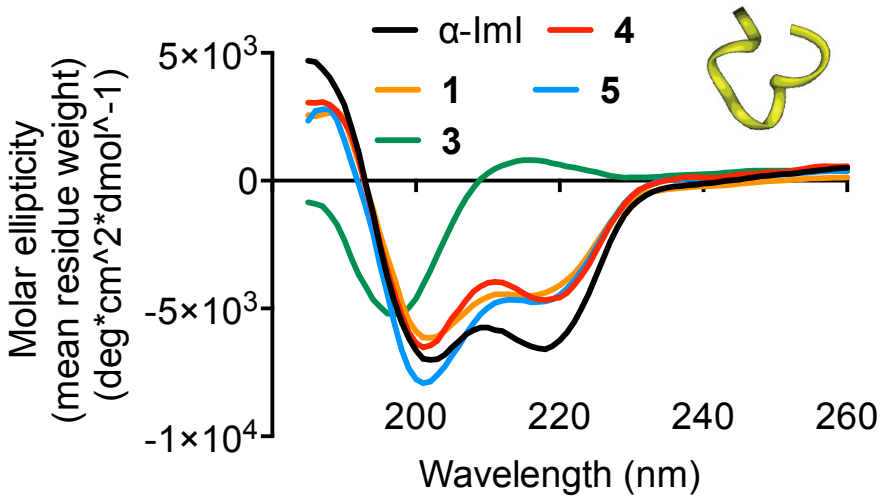


Figure S6. CD spectrum of wild type α-ImI, azide **1**, alkyne polylysine dendron **3** and α-ImI dendrimers **4&5**

NMR analysis

NMR samples contained ~ 0.1 mM peptide in 5% D_2O/95%H_2O at pH ~ 3.5. 1D ^1H NMR spectra and 2D ^1H-^1H TOCSY (mixing time = 80 ms), NOESY (mixing time = 350 ms), and ^1H-^{13}C HSQC NMR spectra were acquired on a Bruker AVANCE spectrometer equipped with a cryogenically cooled probe, operating at a nominal 1H frequency of 900 MHz. Spectra were acquired at 298 K and the temperature was maintained at ± 0.1 K. Assignments were made using all 2D spectra in the program Xeasy.[4]

Table S2. Assigned NH, αH and Cα chemical shifts for α-ImI

Residue	NH	αH	Cα
1.		3.883	43.430
2.	8.829	4.762	4.776
3.	8.384	4.385	57.450
4.	7.946	4.541	56.900
5.	7.970	5.139	50.590

6.		4.354	65.220
7.	8.504	4.354	56.220
8.	8.115	4.417	58.200
9.	8.159	4.134	55.720
10.	7.846	4.480	58.270
11.	7.677	3.794	57.230
12.	7.930	4.535	58.340

Table S3. Assigned NH, αH and Cα chemical shifts for 2xImI-PEG(9)-D **4**. Some Cα chemical shifts could not be obtained as these signals were suppressed by water resonance in ^{1}H-^{13}C HSQC spectrum.

	NH	αH	Cα
1.	8.189	3.595	41.16
2.	8.538	4.776	
3.	8.292	4.347	57.56
4.	7.870	4.517	57.40
5.	7.943	5.125	50.40
6.		4.299	65.05
7.	8.410	4.393	56.20
8.	8.106	4.412	58.35
9.	8.199	4.113	55.74
10.	7.820	4.469	58.09
11.	7.673	3.748	57.34
12.	7.904	4.542	58.31

Table S4. Assigned NH, αH and Cα chemical shifts for 4xImI-PEG(9)-D.

	NH	αH	Cα
1.	8.187	3.441	41.37
2.	8.557	4.727	
3.	8.295	4.351	57.80
4.	7.838	4.509	57.94
5.	7.915	5.140	49.90
6.		4.388	64.96
7.	8.433	4.296	56.08
8.	8.102	4.415	58.34
9.	8.148	4.124	55.75
10.	7.807	4.452	58.09
11.	7.667	3.721	57.51
12.	7.890	4.507	58.28

Ac-AChBp protein expression and purification

Ac-AChBP was expressed as described previously.[5] The protein was purified using affinity chromatography (HF® Ni^{2+} Sigma) followed by gel filtration (Superdex 200 16/600, GE Healthcare), which showed a single, monodisperse peak eluting at a column volume corresponding to that predicted for pentameric Ac-AChBP (data not shown). The purified protein was characterized by 1D SDS-PAGE (Figure S6) and was concentrated to a final concentration of 0.5 mg/mL prior to use.

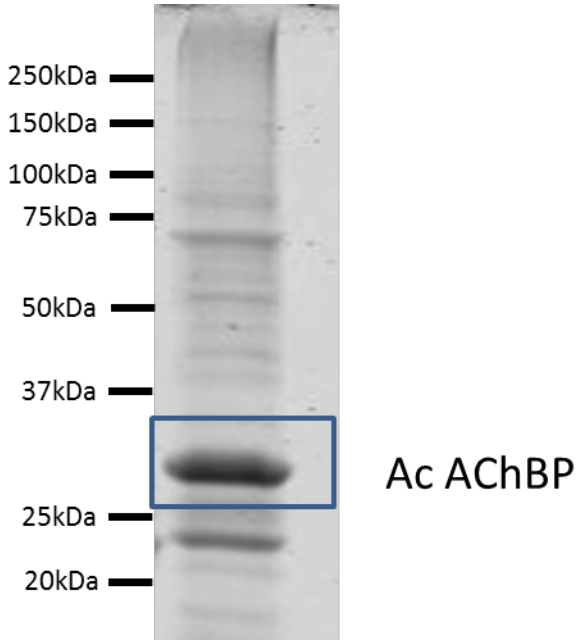


Figure S7. 1D SDS-PAGE of Ac-AChBP

SPR of α-ImI analogues with Ac-AChBP

SPR spectroscopy was performed on a Biacore T200 instrument (GE Health, Australia). Ac-AChBP was immobilized on a CM5 chip via an amino-coupling procedure. In brief, after the activation of surface carboxyl groups using a 1:1 mixture of EDC (1 M) and NHS (0.25 M), 100 µg/mL of Ac-AChBP was dissolved in 0.1M sodium acetate (pH 4.0) and injected across the chip surface at a flow rate of 10 µL/min for 7 min. Then, an injection of 1 M ethanolamine followed to block the chip surface. Approximately 10,000 response units (RUs) of Ac-AChBP was immobilized on the chip. A deactivated flow cell was used as a blank reference. Compounds **1**, **4**, **5** and α-ImI (0.16-20 µM, in 5-fold dilutions) in running buffer (PBS) were injected across the chip at 30 µL/min for 180s to determine their binding to Ac-AChBP. The dissociation period was set up for 180s and assays were repeated 3 times at 25 °C. The reference flow cell response was subtracted from all data generated in the analysis. Kinetic and affinity analysis was carried out using Biacore T200 Evaluation software 2.0 (GE Health, Australia), and a 1:1 binding model was applied preferentially.

The determination of dissociation constant, association constant and binding affinity was performed according to the reference.[6] GraphPad Prism (GraphPad version 6.0, San Diego, CA, USA) was used to generate final figures.

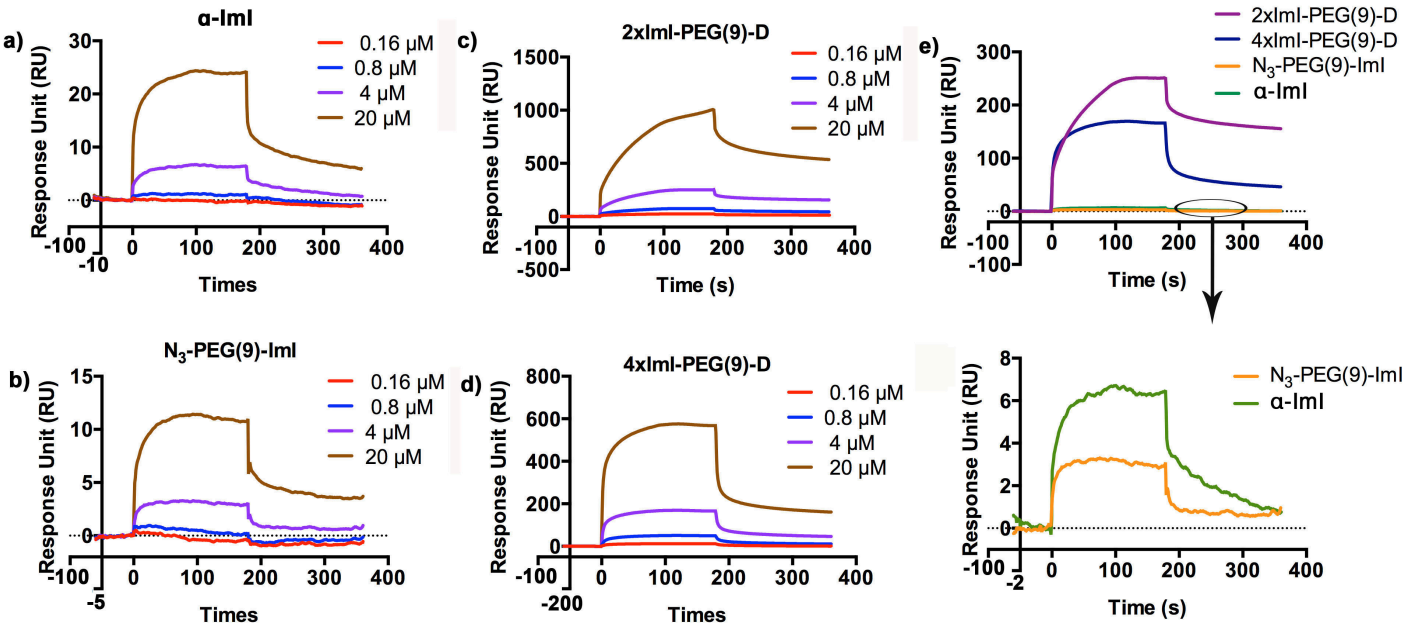


Figure S8. Surface plasmon resonance experiments on Ac-AChBP for a) α-ImI, b) azido-PEG(9)-ImI **1**, c) 2xImI-PEG(9)-D **4** and d) 4xImI-PEG(9)-D **5** in a concentration range of 0.16-20 μM; (e) Binding response at 4 μM α-ImI, azido-PEG(9)-ImI **1**, 2xImI-PEG(9)-D **4** and 4xImI-PEG(9)-D **5**

Binding distance calculation

To estimate the linker length in dimer, the linker was fully drawn in chemoffice with all bonds trans and printed out. By measuring the lengths of one C-C bond and the whole linker from end to end with a ruler, we converted the linker length to Angstroms via the relationship between the C-C length and the actual C-C bond distance (1.54 Å), by which the maximum length of the linker can be obtained.

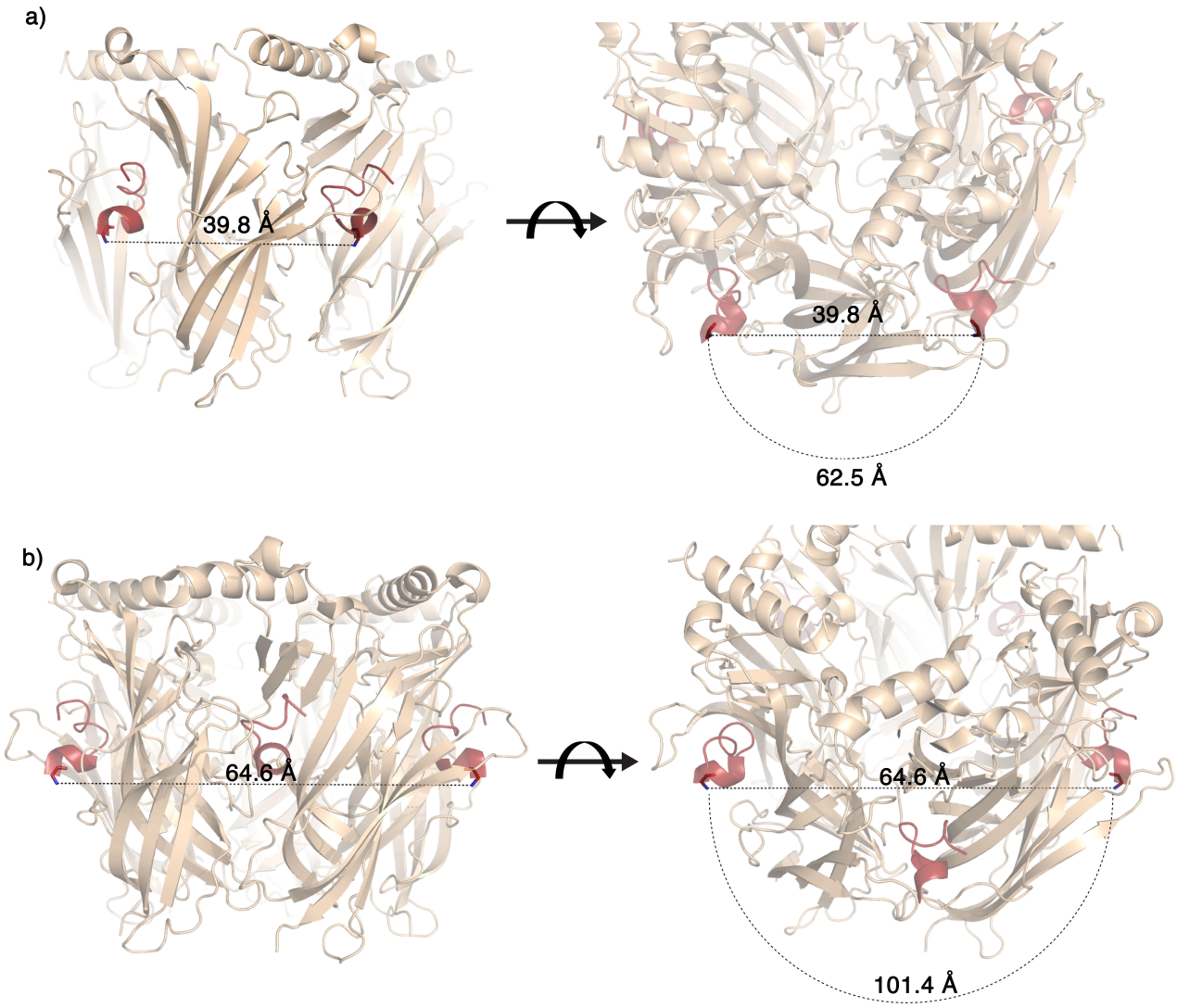


Figure S9. Side and top view of the Ac-AChBP-ImI co-crystal structure (PDB code: 2BYP).[7] The linker is attached to the N-termini of ImI (highlighted as sticks). a) The distance between two adjacent ImI N-termini is 39.8 Å. Assuming a spherical approach, the minimum distance for the linker to span across two adjacent sites would be 62.5 Å (considering 39.8 Å as the diameter, total circumference for the circle would be 125 Å); b) The distance between two ImI N-termini binding to two immediately non-adjacent sites is 64.6 Å. Again assuming a spherical approach, the minimum distance to be spanned the linker to bind across these sites would be 101.4 Å (diameter of 64.6 Å and total circumference 202.8 Å). It is notable that the angle between the linker and bound ImI is shallower for the adjacent compared to non-adjacent site binding, which is expected to dis-favour binding to non-adjacent sites.

Electrophysiology

cRNA preparation. Plasmid DNAs encoding human(h) α3, α7, α9, α10, and β2 nAChR subunits were linearized with appropriate restriction enzymes, and cRNA was synthesized in vitro using a SP6 or T7 *in vitro* transcription kit (mMessage mMachine; Ambion, Foster City, CA).

Oocyte preparation and microinjection. Stage V-VI oocytes were obtained from Xenopus laevis and defolliculated using 1.5 mg/ml collagenase Type II (Worthington Biochemial Corp., Lakewood, NJ) in OR-2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES) at pH 7.4. Oocytes were injected with 5 ng cRNA of each subunit for the nAChR subtypes to be tested and were incubated at 18ºC in sterile ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM MgCl2 and 5 mM HEPES at pH 7.4) supplemented with 5 % FBS, 50 mg/L gentamicin (GIBCO, Grand Island, NY) and 100 μg/units/mL penicillin-streptomycin (GIBCO, Grand Island, NY). Electrophysiological recordings were carried out 2–7 days after microinjection.

Electrophysiological recordings and data analysis. Two-electrode (virtual ground circuit) voltage clamp recordings from oocytes were carried out at room temperature (21-23°C) using a GeneClamp 500B amplifier (Molecular devices Corp., Sunnyvale, CA) and a holding potential of −80 mV. Voltage-recording and current-injecting electrodes were pulled from borosilicate glass (GC150T-7.5, Harvard Apparatus Ltd., Holliston, MA) and had resistances of 0.3–1 MΩ when filled with 3 M KCl. ¬A continuous push/pull syringe pump perfusion system (KD Scientific, Holliston, MA) was used to perfuse oocytes with ND96 at a rate of 2 ml/min. nAChR–mediated currents were evoked by application of acetylcholine (ACh) at a rate of 2 ml/min via the perfusion system. Currents were elicited by 100 μM, 50 μM and 20 μM ACh for hα7, hα9α10 and hα3β2, respectively. Washout periods of 3-4 mins between applications of ACh were used. Oocytes were incubated with peptides for 5 mins before ACh was co-applied. Peak ACh-evoked current amplitude was recorded before and after peptide incubation using pClamp 9 software (Molecular Devices Corp., Sunnyvale, CA).

All electrophysiological data were pooled (n = 3 to 5 for each data point) and represent means ± standard error of the mean (SEM). Data sets were compared using an unpaired Student's t-test. Differences were statistically significant when $p < 0.05$. Concentration-response curves for α-ImI, N_3-PEG(9)-ImI, 2xImI-PEG(9)-D and 4xImI-PEG(9)-D were obtained by plotting averaged relative peak current amplitude values (I/Icontrol) as a function of peptide concentration and fitting the relationship with the Hill equation, $I = Icontrol\{[CTX]n/(IC50n + [CTX]n)\}$, where Icontrol is the maximum peak current amplitude, [CTX] the peptide concentration, n the Hill coefficient, and IC_{50} the half-maximal inhibitory concentration of the antagonist.

FLIPR analysis

Functional activity of α-ImI, azido-PEG(9)-ImI **1** and dendrimeric ImI **4&5** at α7 nAChR endogenously expressed in human SH-SY5Y neuroblastoma cells was assessed using a high-throughput FLIPR^{Tetra} (Molecular Devices, Sunnyvale, CA) fluorescent plate reader assay. In brief, SH-SY5Y cells were plated at a density of 150, 000 cells/well on black-walled, clear bottom 384-well imaging plates (Corning) 48 hours prior to the assay. Cells were loaded for 30 minutes at 37 °C with Calcium-4 No Wash kit (Molecular Devices, Sunnyvale, CA) in physiological salt solution (PSS; composition NaCl 140 mM, glucose 11.5 mM, KCl 5.9 mM, $MgCl_2$ 1.4 mM, NaH_2PO_4 1.2 mM, $NaHCO_3$ 5 mM, $CaCl_2$ 1.8 mM, HEPES 10 mM). Fluorescence readings were obtained with cooled CCD camera at excitation 470 – 495 nM and emission 515–575 nM. After 10 baseline fluorescence reads, compounds including α-ImI, azido-PEG(9)-ImI **1** and dendrimeric ImI **4&5** diluted in PSS containing 10 μM PNU120596 were added and fluorescence readings were obtained every second for 300 seconds. Following addition of the α7 nAChR specific agonist choline (30 μM), responses were measured every second for a further 300 seconds. Data is representative of 3–5 independent experiments with at least n=3 replicates per concentration

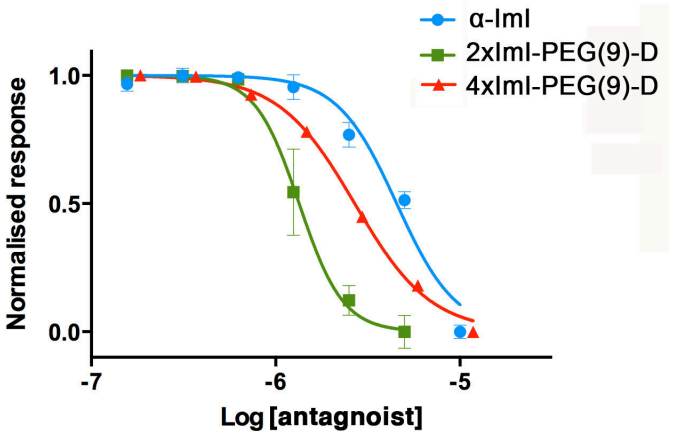


Figure S10. Functional data of the ImI dendrimers (**4-5**) and α-ImI on α7 nAChR receptor. The functional EC_{50} values were investigated by measuring the stimulation of Ca^{2+} responses from SH-SY5Y cells expressing α7 nAChR receptor. Representative concentration-response curves performed in triplicate for each dendrimer at the α7 nAChR receptor.

Table S5. IC_{50} and pIC_{50} values for stimulation of Ca^{2+} response from SH-SY5Y cells expressing α7 nAChR receptor. All values are means ± SEM of 3 separate experiments (performed in triplicate).

Entry	Analogue	IC_{50} (uM)	pIC_{50}
	α-ImI	5.0	5.2 ± 0.1
4	2xImI-PEG(9)-D	1.3	6.0 ± 0.4
5	4xImI-PEG(9)-D	2.8	5.7 ± 0.4

References:

(1) Alewood, P.; Alewood, D.; Miranda, L.; Love, S.; Meutermans, W.; Wilson, D. *Method Enzymol* **1997**, *289*, 14.

(2) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.

(3) Sawicki, E.; Stanley, T. W.; Hauser, T. R.; Barry, R. *Anal. Chem.* **1959**, *31*, 1664.

(4) Bartels, C.; Xia, T. H.; Billeter, M.; Guntert, P.; Wuthrich, K. *J. Biomol. NMR* **1995**, *6*, 1.

(5) Celie, P. H. N.; van Rossum-Fikkert, S. E.; van Dijk, W. J.; Brejc, K.; Smit, A. B.; Sixma, T. K. *Neuron* **2004**, *41*, 907.

(6) Cooper, M. A.; Williams, D. H. *Anal. Biochem.* **1999**, *276*, 36.

(7) Hansen, S. B.; Sulzenbacher, G.; Huxford, T.; Marchot, P.; Taylor, P.; Bourne, Y. *Embo. J.* **2005**, *24*, 3635.