Inhibition of the norepinephrine transporter with χ-conotoxin dendrimers

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Abstract: Peptide dendrimers are a novel class of macromolecules of emerging interest with the potential of delayed renal clearance due to their molecular size and enhanced activity due to the multivalency effect. Difficulties in the synthesis of homogeneous dendrimers carrying well-structured peptides have largely hampered progress in this field. In this work, an active analogue of the disulfide rich chi-conotoxin χ-MrIA, a norepinephrine reuptake (NET) inhibitor, was grafted onto a polylysine dendron. Dendron decoration was achieved by employing copper catalyzed alkyne-azide cycloaddition (CuAAc) in combination with the use of hydrophilic PEG linkers, leading to homogenous 4- and 8-mer χ-MrIA dendrimers with molecular weights from 8 KDa to 22 KDa. These dendrimers were investigated for their impact on peptide secondary structure, *in vitro* functional activity and potential anti-allodynia function *in vivo*. NMR studies showed that the χ-MrIA-peptide analogue tertiary structure was identical to the parent peptide in the two dendrimers. In a functional norepinephrine transporter reuptake assay, χ-MrIA dendrimers showed slightly increased potency relative to the azido-PEGylated χ-MrIA analogues but no improvement compared to the parent peptide χ-MrIA. In contrast to χ-MrIA, no anti-allodynic action was observed when χ-MrIA dendrimers were administered intrathecally in a rat model of neuropathic pain, suggesting that the larger dendrimer structure is unable to diffuse through spinal column tissue to reach the NET transporter.

Keywords: conotoxin, dendrimer, Click reaction, χ-MrIA, norepinephrine reuptake (NET) inhibitor

Introduction

Dendrimers with polyvalent structures have been extensively investigated in biomedicine in the past few decades.[1, 2] In particular, they are widely regarded as useful carriers for small molecule drugs and gene delivery though bioactive peptides have been less exploited.[3, 4] Peptides by themselves represent a large class of bioactive molecules and have emerged as prospective drug candidates for various diseases.[5, 6] By integrating the merits of dendrimers with bioactive peptides, dendrimeric peptides have produced diverse synergistic effects, including a multivalency effect that enhances the avidity of the peptide ligand while delaying renal clearance due to its increased molecular size.[7, 8, 9, 10] Many synthetic approaches are now available for generating peptide dendrimers either in a stepwise manner (divergent) or by ligation of pre-purified segments (convergent).[11, 12] Nevertheless, the synthesis of fully decorated dendrimers with multiple copies of the peptide ligand, particularly with disulfide bond-containing peptides, has proven to be challenging.[13] Several conjugation strategies such as peptide coupling chemistry,[14] thiol-maleimide chemistry,[15] copper catalyzed alkyne-azide cycloaddition[10] and oxime ligation[16] have been applied in the design and synthesis of oxidatively folded peptide dendrimers, though at most tetramer decorated dendrimers have emerged with incomplete decoration[14] and disulfide bond shuffling issues have been observed.[15] Fully decorated dendrimers containing more than 4 copies of cysteine-rich peptides remain thus far unreported.

Disulfide bonds are a common posttranslational modification and they are functionally important in bioactive peptides and proteins.[17] Disulfide bridges are highly prevalent in peptide toxins secreted in animal venoms, e.g. conotoxins.[18] Conotoxins are cysteine-rich bioactive peptides from cone snails that act on a wide variety of ion channels, G-protein coupled receptors (GPCR's) and transporters with exquisite potency and selectivity.[19, 20, 21] Of particular interest is χ-conotoxin MrIA (χ-MrIA), which contains 2 disulfide bonds arranged in a "ribbon" structure (1-4, 2-3 connectivity) and acts as a selective inhibitor of the norepinephrine transporter (NET).[22] These χ-conotoxins has been translated into the clinic for the treatment of chronic neuropathic pain.[23, 23, 24] In addition, NET inhibitors have the potential to treat a number of neurological conditions, such as depression,[25] schizophrenia,[26] anxiety[27] and other psychotic disorders.[26] An alanine scan[28] of C-terminally amidated χ-MrIA (NGVCCGYKLCHOC-NH_2, 1-4, 2-3 connectivity) and intensive SAR work[20] has shown that the NET-modulating pharmacophore is mainly located within the inter-cysteine GYKL loop sequence and this has eventually lead to the development of peptidomimetics of χ-MrIA.[29] It was evident from this earlier work that any

small structural changes in the pharmacophore significantly interfere with the biological activity.[20] Thus to maintain dendrimers with bioactivity demands that the peptide secondary structure remains undisturbed. The previous structure–activity relationship (SAR) studies also revealed that the N-terminal of χ-MrIA is not located in the pharmacophoric region, and thus provides an ideal ligation site for linkage to a dendrimer platform.[28] Furthermore, it was shown that hydroxyproline (position 11) can be substituted by proline[28] and the asparagine residue responsible for unwanted aspartimide formation[30] can be truncated[31] without altering the hNET-bioactivity of the peptide.

The clinical χ-MrIA analogue (Xen2174) was developed for intrathecal injection and its duration of action was largely determined by the clearance rate from the IT space into the plasma.[32] Given the polyvalent structure and large molecular size, a dendrimeric formulation of χ-MrIA was anticipated to be an interesting synthetic target to modulate the pharmacokinetic properties. In this study, we undertook the synthesis of χ-MrIA dendrimers of differing size PEG spacers with molecular weights ranging from 8 KDa to 22 KDa and further investigated their structural and functional features. Initially, χ-MrIA dendrimers carrying 4 and 8 copies of the χ-MrIA analogues ([Pro]^{11}MrIA[2-13], **1**) were successfully synthesized through the copper-catalyzed alkyne azide cycloaddition (CuAAc) reaction, based on the alkyne modified polylysine (PLL) dendrons (**2** and **3,** Figure 1**)** and azido-PEG-chain modified χ-MrIA analogues [N_3-PEG(9 or 24)-[Pro]^{11}MrIA[2-13]-NH_2 (**4** and **5,** Figure 2). The CuAAc reaction is a widely used strategy in peptide conjugation promising high efficiency and high selectivity.[33, 34] Scheme 1 depicts the structure of an octameric χ-MrIA dendrimer **7** containing a lysine dendron core, PEG spacer units and a small charged (Gly-Arg_4-Gly) tail we introduced to facilitate solubility and ease of mass spectrometric evaluation. The flexible polyethylene glycol segments of different lengths, approximately 35Å and 90Å respectively, were introduced between the peptide and dendron to reduce steric bulk and to herewith allow for sufficient dendron loading as well as increasing the solubility of the dendrimeric construct. The χ-MrIA dendrimers (**6**-**8**) were purified and characterized by HPLC coupled ESI-MS to confirm purity and homogeneity. Subsequently, their 3-dimensional structures were determined by 2D NMR spectroscopy and their potency via an *in vitro* NE uptake cell-based assay and an *in vivo* pain model. These studies contribute to our understanding of multivalent peptide dendrimers.

Materials and methods

Materials. All Nα-Fmoc-L-amino acids, Fmoc-L-Lys(Fmoc), N_3-PEG-(9)-COOH, N_3-PEG-(24)-COOH and HBTU were purchased from IRIS Biotech (Marktredwitz, Germany). Rink Amide resin, DIEA, trifluoroacetic acid (TFA), DMF and piperidine were purchased from Auspep (Melbourne, Auatralia). DCM, ACN and diethyl ether were purchased from Merck (Melbourne, Australia). 5-azido-pentanoic acid was purchased from BACHEM Feinchemikalien AG (Bubendorf, Switzerland). HATU was imported from GL Biochem Ltd. (Shanghai, China). TBTA, 5-hexynoic acid, sodium azide, copper sulfate, sodium ascorbate, cooper iodide, copper wires, DMSO, Triisopropylsilane as well as all other reagents and solvents, were purchased from Sigma-Aldrich. (^{3}H)NE, 96-well plate and Optiphase Supermix scintillant were all from Perkin Elmer. Water measuring 18.2 MΩ (ELGA, Melbourne, Australia) 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.

HPLC analysis and purification. 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 Agilent Zorbax 300SB-C_{18} analytical column (300 Å, 5 μm, 150 mm × 4.6 mm). Data was recorded and processed with Shimadzu Class VP software. The absorbance was monitored by a UV detector at 214 nm and 254 nm, respectively. The HPLC solvent system was made as follows: solvent A was 0.05 % TFA in millpore water, while solvent B was 0.043% TFA in ACN/H_2O (v/v: 90/10). For the peptide purification, two types of columns were used including GRACE-Vydac C_{18} column (300 Å, 10 μm, 250 mm × 22 mm) and Agilent Zorbax C_{18} column (300 Å, 10 μm, 250 mm × 10 mm). The purification was carried out by preparative RP-HPLC system 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.

Electrospray Mass Spectrometry (ESI-MS). PESCIEX API150EX mass spectrometer was coupled with HPLC to perform the peptide analysis. The Mass Spectrometry was operating in the positive ion mode with a declustering potential (DP) of 20 V, a focusing potential (FP) of 220 V and a turbospray heater temperature of 350°C.

Peptide Concentration Assessment. Peptide concentration was calculated based on peak area detected at 214 nm by analytical HPLC. A peptide (χ-MrIA) with known concentration identified

by amino acid analysis was used as the standard peptide. Molecular extinction coefficients were calculated according to the increments set up by Buck et al[35] for the standard and sample peptides. By comparing the absorption data of the standard and samples.

General protocols for Fmoc-SPPS synthesis. Peptides and dendrimers were assembled by Fmoc/tBu-based manual SPPS on a Rink amide resin (loading 0.71 mmol/g) using the HBTU/DIEA *in situ* neutralization protocol[36] to activate the standard residues; treatment with 30% piperidine/DMF (2 × 1 min) was used for Fmoc deprotection. Coupling was 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.[37] For the couplings onto a proline residue, the efficiency was measured by the Isatin test.[38] N_3-PEG(9)-COOH, N_3-PEG(24)-COOH, and 5-hexynoic acid were coupled with 1.5 or 2 equiv of excess for a minimum of 1h. Cleavage of the peptide/dendrimer from resin was performed under standard conditions (TFA : triisopropylsilane : water, 90 : 5 : 5, 3 h). After the cleavage was complete, 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% Acetonitrile/water +0.0425%TFA and then lyophilized.

Synthesis of first- and second- generation alkyne-dendrons 2 and **3 [**$GlyArg_4Gly$-$Lys[Lys]_2[CO\text{-}(CH_2)_3\text{-}C{\equiv}CH]_4$, **2**; $GlyArg_4Gly$-$Lys[Lys]_2[Lys]_4[CO\text{-}(CH_2)_3\text{-}C{\equiv}CH]_8$, **3**] The sequence of GRRRRG was first assembled to the Rink amide resin. Then 4 equiv of excess Fmoc-L-Lys(Fmoc) acid per amine group was added to the coupling system to achieve dendrons with 4 and 8 final terminal amino groups. After that, 5-hexynoic acid, activated by 0.5 M HATU/DIEA, was coupled to the Lys-dendrons for 24h with 2 equiv excess per dendron-branch. After TFA cleavage, the crude products were purified with a preparative C_{18} column with 1 %/min linear gradient of 10-60 % buffer B at a flow rate of 15 mL/min.

Synthesis and oxidation of χ-MrIA analogues 4 and **5** [N_3-PEG(9)-CO-NH-Gly-Val-Cys-Cys-Gly-Tyr-Lys-Leu-Cys-His-Pro-Cys-NH_2, **4**; N_3-PEG(24)-CO-NH-Gly-Val-Cys-Cys-Gly-Tyr-Lys-Leu-Cys-His-Pro-Cys-NH_2, **5** (Cysteine connectivity: 1-4, 2-3)] Reduced χ-MrIA analogues were synthesized through the standard Fmoc-SPPS method as above and the azido PEG spacer was attached to the amine group of the N-terminal glycine residue. Specifically, Fmoc-Cys(Acm)-OH were coupled in position Cys^3 and Cys^{12} while Fmoc-Cys(Trt)-OH were used in

position Cys^4 and Cys^9. N_3-PEG(9)-COOH and N_3-PEG(24)-COOH activated by HATU were coupled to the N-terminal glycine residue overnight. After TFA cleavage, the crude reduced peptides were dissolved in 0.1 M ammonium bicarbonate buffer at pH 8 to form the first disulfide bond between Cys^4 and Cys^9 employing air as oxidant. The oxidation products were subjected to preparative RP-HPLC system. The purified peptides were then treated with iodine (1 mM) to remove the Acm protecting group and form the second disulfide bond between Cys^3 and Cys^{12} simultaneously. After 1h, 0.1 M ascorbic acid was added to quench the reaction. The fully oxidized products were purified with a preparative C_{18} column with 1 %/min linear gradient of 20-60 % buffer B at a flow rate of 15 mL/min.

In-solution click reaction of alkyne-dendron (2, 3) and azido-PEG(n)-[Pro]^{11}MrIA[2-13] (4, 5) **[**$GlyArg_4Gly$-$Lys[Lys]_2[CO$-$(CH_2)_3$-1,2,3-triazole-PEG(9)-CO-NH-Gly-Val-Cys-Cys-Gly-Tyr-Lys-Leu-Cys-His-Pro-Cys-$NH_2]_4$, **6**; $GlyArg_4Gly$-$Lys[Lys]_2[Lys]_4[CO$-$(CH_2)_3$-1,2,3-triazole-PEG(24)-CO-NH-Gly-Val-Cys-Cys-Gly-Tyr-Lys-Leu-Cys-His-Pro-Cys-$NH_2]_4$, **7**; $GlyArg_4Gly$-$Lys[Lys]_2[CO$-$(CH_2)_3$-1,2,3-triazole-PEG(24)-CO-NH-Gly-Val-Cys-Cys-Gly-Tyr-Lys-Leu-Cys-His-Pro-Cys-$NH_2]_4$, **8** [cysteine connectivity: 1-4, 2-3)] $CuSO_4$ and sodium ascorbate were dissolved in water at a concentration of 0.1 M, respectively. Alkyne-dendrons **2** or **3 (**Dendron concentration 5 μM**)** were dissolved in DMF. Azido-PEG(n)-[Pro]^{11}MrIA[2-13] (**4** or **5**; 2 equiv/branch) was weight and the solution of $CuSO_4$ (0.2 equiv/branch) and TBTA ligand (0.2 equiv/branch) was added. The mixture was degassed with nitrogen for 15 min and the solution of sodium ascorbate (0.5 equiv/branch) was added to the sealed reaction vessel to initiate the click reaction. The reaction was performed overnight at room temperature and the resulting reaction products were purified with a Zorbax semi-preparative C_{18} column with 0.5 %/min linear gradient of 20-60 % solvent B at a flow rate of 7 mL/min.

NMR analysis. Samples for NMR spectroscopy contained ~ 0.1 mM peptide dendrimer in 5% D_2O/95%H_2O at pH around 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 acquire at 298 K and the temperature was maintained at ± 0.1 K.

NE uptake inhibition assay. COS-7 Cells were transiently transfected with NET cDNA using 2 μg DNA and 10 μl FuGENE per 10^6 cells at the confluency of 90 %. After 24h culture, 10,000 cells were evenly distributed per well in a 96-well plate for another 24 hours incubation. An average cell count of approximately 50,000 cells per well was counted before uptake assays. A fixed concentration of (^{3}H)NE of 30 nM (40.5 Ci/mmol) and increasing concentrations of peptide analogues were added into 96-well plates and incubated at 37 °C for 10 min, followed by two gentle washes with 100 μl 25 mM HEPES buffer to remove excess (^{3}H)NE. The concentration of χ-MrIA dendrimers was calculated based on the dendrimer concentration rather than the concentration of the attached χ-MrIA ligand. Cells were then lysed using 50 μl of 0.1 M NaOH with gentle shaking for 60 min. Lysed cells were transferred to the flexible 96-well plate with Optiphase Supermix scintillant for measurements. Each experiment was performed in triplicate and repeated three times.

Anti-allodynia and motor side effect testing in a rat model of neuropathic pain. Experiments were performed on male Sprague Dawley rats (n = 31) weighing 252 +/- 15g on the day of drug injections (ARC WA, Perth Australia). Rats were caged in groups of two or three under standard conditions with a 12 h light/dark cycle and free access to food and water. A four to five day acclimatization period was allowed in the housing facility prior to performing any procedures. All procedures were approved by the Royal North Shore Hospital/University of Sydney Animal Ethics Committee and followed the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013). Baseline measurements of mechanical paw withdrawal threshold (PWT) and rotarod performance were taken prior to nerve ligation surgery. For mechanical PWT rats were acclimatized to clear Perspex chambers, with mesh flooring prior to testing. A series of calibrated von Frey filaments 0.4-15.1g (Stoelting, USA) were presented using the up-down method.[39] If the rat responded to all the filaments, a minimal score of 0.2g was assigned. If the rat did not respond to any filaments a maximal score of 15g was assigned. An accelerating rotarod (Ugobasile, Italy) was used to measure motor co-ordination. Rats were trained to run on the rotarod before baseline measurements were taken. The rats were placed on the rotarod and latency to fall was recorded in seconds, with a maximal cut off of 300 seconds.

Partial ligation of the left sciatic nerve (PNL) was performed on each animal.[40] Under isoflurane anaesthesia (1.5-4% in oxygen) an incision was made through the skin and muscle at mid-thigh

level of the lateral left hind leg. The sciatic nerve was gently isolated from the surrounding connective tissue using blunt dissection, and one half to two-thirds of the nerve was ligated with 6/0 silk suture (Ethicon, USA). Muscle and skin layers were closed using 4/0 and 3/0 silk suture (Ethicon, USA) respectively. Intrathecal catheters were placed under isoflurane anaesthesia (1.5-4% in oxygen) seven to eight days after PNL surgery. A skin incision was made on the dorsal midline in the lumbar area. Polyethylene tubing was inserted into the intrathecal space through an 18g needle placed in between the fourth and fifth lumbar vertebrae. The tubing was advanced three to four centimetres cranially in the intrathecal space. Correct placement was indicated by tail and/or leg twitch when the needle pierced the intrathecal space, and the presence of cerebrospinal fluid in the tubing when gently aspirated after placement. The distal end of the catheter was tunneled subcutaneously and exteriorized at the level of the neck to prevent self-removal. After catheter placement the rats were housed individually until the drug injections and repeat behavioural tests were performed (maximum of four days).

On the day of the experiment acclimatization and baseline behavioural measurements were performed 30 minutes prior to drug injections and repeated immediately prior to injections (time=0 measurement). Drugs were dissolved in 0.9% saline to achieve the required concentration, injected in 10μL volumes and flushed through the catheter with 25μL of 0.9% saline. A 20nmol dose of each dendrimer (4xMrIA-PEG(9) n = 5, 4xMrIA-PEG(24) n = 5, 8xMrIA-PEG(24) n = 6) was tested. A 20 nmol dose of MrIA (n=8) was used as a positive control, and 0.9% saline (n=7) acted as a negative/vehicle control. Behavioural measurements (von Frey and rotarod) were repeated hourly for four hours after drug injections. Animals were also observed for behavioural side effects throughout the experiment. The experimenter was blinded to the drug treatment. Mechanical PWT and rotarod data were analyzed with GraphPad Prism 5 (GraphPad Software, San Diego, California, USA) using repeated measures ANOVA, followed by Bonferonni post hoc testing when ANOVA was significant.

Results and Discussion

Synthesis of alkyne polylysine dendrons and azido χ-MrIA peptides. The lysine-based dendrons **2** and **3** and χ-MrIA analogues **4** and **5** possessing alkyne and azido functional groups were synthesized using Fmoc solid-phase peptide synthesis (SPPS).[41] The dendron scaffold was assembled from bis-Fmoc-Lysine as described by Tam,[42] taking advantage of the two amino groups in each lysine molecules which constitute branching points for exponential growth.[11] $GlyArg_4Gly$ residues[43] were introduced as a hydrophilic tail to the Rink amide resin to increase the solubility of the alkyne modified dendrons and facilitate the MS ionization. By addition of further lysine layers we were able to obtain dendrons with 4 and 8 amino groups on the surface, which were fully converted into alkyne groups through conjugation with 5-hexynoic acid (Figure 1). The crude compounds (**2** and **3**) were subjected to semi-preparative RP-HPLC purification to give >90% pure alkyne dendrons (**2** and **3)**, as confirmed by ESI-MS (Figure S1).

Previous work has demonstrated that the replacement of hydroxyproline with proline residues in χ-MrIA is tolerated without loss of binding affinity toward NET.[20] Moreover, a truncated analogue χ-MrIA with residue asparagine-1 deleted maintained its 3D structure and binding affinity comparable to amidated χ-MrIA.[20, 31] To avoid the formation of aspartimide,[30] the truncated χ-MrIA analogue **1**, ($[Pro]^{11}$MrIA[2-13], GVCCGYKLCHPC-NH_2, 1-4, 2-3 connectivity) was used to build the azido-modified χ-MrIA moiety **4** and **5**. The azido group was introduced to the N-terminus of **1** by coupling of azido-PEG(9)-COOH or azido-PEG(24)-COOH with chain lengths of approximately 35Å and 90Å respectively. The two PEG chains were introduced to increase the solubility of dendrimeric χ-MrIA and more importantly, to assess their performance in reducing the steric hindrance for sufficient dendron loading. To ensure correct folding of **4** and **5**, pairs of Fmoc-cysteine(Acm)-OH and Fmoc-cysteine(Trt)-OH were utilized in the synthesis to direct regioselective disulfide bond formation (Figure 2).[44] The crude peptides were oxidized stepwise, purified after each oxidation step and then subjected to the RP-HPLC for purification to give >90% pure peptides **4** and **5** (denoted as azido-PEG(9)-$[Pro]^{11}$MrIA[2-13] and azido-PEG(24)-$[Pro]^{11}$MrIA[2-13], respectively) with native (1-4, 2-3) disulfide connectivity. (Figure S2).

Efficient CuAAc Ligation between alkyne lysine dendrons and azido peptides.

The CuAAc reaction possesses many properties that distinguish it from alternative coupling strategies – these include the rapid rate of reaction and its high chemoselectivity.[45] Click reaction was herewith selected as the tool for the synthesis of χ-MrIA dendrimers. This was achieved by coupling of a 2-fold excess of azido-PEG(9)-[Pro][11]MrIA[2-13] peptide **4** per dendrimer arm of the alkyne dendron **2** in the presence of $CuSO_4$ and sodium ascorbate as the reducing reagent for the formation of the catalytic Cu(I). The tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) ligand which stabilizes Cu(I) towards disproportionation and oxidation[46] was also added to the mixture to enhance the Cu(I) catalytic effect. The reaction was monitored by LC ESI-MS and a new peak was detected in the LC trace after overnight reaction whereas the alkyne-dendron (**2**) peak disappeared (Figure S3a, b). The reaction mixture was then subjected to semi-preparative RP-HPLC resulting in a >90% pure tetravalent χ-MrIA dendrimer, referred to as 4xMrIA-PEG(9)-D (**6).** Its identity was confirmed by ESI-MS (Figure 3a, Table 1). In order to investigate the influence of increasing size and copy number on the χ-MrIA dendrimers on pharmacology we attempted to build an 8-mer χ-MrIA dendrimer by click chemistry conjugation of dendron **3** with the azido PEG 9-MrIA analogue **4** which resulted disappointingly in a mixture of partially decorated χ-MrIA dendrimers with 5, 6, and 7 copies of the χ-MrIA moiety (Figure 2b).

The difficulty associated with driving complete decoration with the χ-MrIA analogue possessing the PEG 9 spacer was bypassed by elongating the PEG chain from PEG 9 to PEG 24, where the steric hindrance in the decoration could be reduced with the longer PEG linker. As expected, the fully decorated conjugate of the 8-mer alkyne dendron (**3)** with azido-PEG(24)-[Pro][11]MrIA[2-13] (**5)** was readily obtained (Figure S3e, f), providing a pure octavalent χ-MrIA dendrimer (denoted as 8xMrIA-PEG(24)-D, **7**) with the observed mass consistent with the theoretical one (Figure 3c, Table 1). We also designed the 4-mer χ-MrIA dendrimer (denoted as 4xMrIA-PEG(24)-D, **8**) to compare the impact of PEG length on potency (Figure 3d, S3c, d, Table 1).

2D-NMR structural analysis. NMR is very sensitive to minor local variations in the chemical environment and are widely used to detect structural changes for particular parts of molecules.[47] For peptides it is time-consuming to perform a full 3D solution structure determination, hence the structural changes are routinely determined by comparing the Hα chemical shifts with

chemical shifts derived from random coil values.[10] Here, 1D and 2D-NMR spectroscopy at 900 MHz was employed to characterize the structures of three multivalent χ-MrIA dendrimers (**6-8**) and the truncated analogue [Pro][11]MrIA[2-13] (**1**). For the investigated χ-MrIA dendrimers (**6-8**) only a single set of resonance lines for each attached χ-MrIA moiety was observed in the TOCSY, NOESY and HSQC analysis, indicative of free and independent motion of each χ-MrIA unit in all dendrimers (**6-8)**. We then compared in detail the structural difference between the χ-MrIA dendrimers and monomer by assigning and comparing their secondary αH chemical shifts. This technique is very sensitive for the detection of structure change[48] and the obtained results are shown in figure 4a (Table S1-4). These experiments revealed that χ-MrIA-NH_2[31] and truncated [Pro][11]MrIA [2-13] (**1**) monomers have very similar αH secondary shifts especially in the very important GYKL pharmacophore turn region (Figure 4b), confirming that proline substitution has little effect on pharmacophore structure. Additionally, all the copies of χ-MrIA analogue presented on dendrimers showed no significant secondary αH shift differences, indicative of no change in their structures when compared to the truncated monomer **1** or the χ-MrIA-NH_2 (Figure 4a). Hence, we can conclude that the dendrimer decoration has no influence on the secondary structure of χ-MrIA peptide-portion and in particular the GYKL-pharmacophore region of the peptide. The χ-MrIA dendrimers **6-8** maintain unaltered peptide structures that are free to interact with NET independently of each other.

In *vitro* and in *vivo* functional analysis. The in *vitro* functional activity of χ-MrIA dendrimers **6-8** and their monomer analogues **4** and **5** were evaluated using a functional human norepinephrine transporter assay. As shown in Table 1, the introduction of the azido-PEG chain generated active χ-MrIA analogues **4** and **5** with less potency than χ-MrIA-NH_2 (Figure 5a, b). This loss of affinity was most prominently observed for the PEG(24) peptide **5** compared to the PEG(9) peptide **4**. This may indicate an interference of the long N-terminal PEG chain on transporter binding. In contrast, the χ-MrIA dendrimers **6** and **7** were ~3-fold and ~40-fold more potent than their monomeric counterparts, suggesting that the tethered χ-MrIA analogues were accessible at the NET binding and possibly cause a slight multivalency. Also, dendrimers **6** and **7** possessed comparable IC_{50} values with χ-MrIA-NH_2, and thus can be used to mimic the bioactivity of χ-MrIA-NH_2. Further analysis revealed that for a small dendron like **2** with only four χ-MrIA molecules, a short linker of PEG 9 is sufficient (see Table 1, **6**) to obtain full bioactivity. For the larger dendron with eight peptide molecules surrounding the dendron sphere

(**7**), the PEG(24) resulted in optimum activity equipotent to the χ-MrIA-NH_2. On the other hand if the chosen PEG chain is too long one might expect an increase of flexibility potentially affecting the bioactivity. The elongation of the PEG chain negatively affects the functional activity of **8** (small dendron **2** with a long PEG(24) chain) compared to **6** (small dendron **2** with a short PEG(9) chain). These findings suggest that for the design of peptide dendrimer that are functional, both dendrimer size and linker length need to be optimised.

After establishment of the *in vitro* activity of the optimized χ-MrIA dendrimers **6-8**, we were interested in investigating how the dendrimeric structure might influence the pharmacokinetics in a rat pain model of neuropathic pain, which has been extensively used for the validation of the duration of action of χ-MrIA analouges.[20] The monomer χ-MrIA-NH_2 was potent and remained anti-nociceptive for up to 3 days after intrathecal administration.[49] Given their highly branched architectures and increased molecular weights, we had anticipated that the larger dendrimeric χ-MrIA molecules may show a much more sustained duration in an *in vivo* pain model. To conduct the in *vivo* test, partial ligation of the sciatic nerve (PNL) was performed, which results in several symptoms indicative of neuropathic pain, including marked mechanical allodynia. Mechanical paw withdrawal threshold (PWT) measured using calibrated von Frey filaments reduced from 14.6±1.3g prior to surgery to 1.0±1.2g prior to drug injection at 10-11 days after PNL surgery. χ-MrIA dendrimers (**6-8**) and amidated χ-MrIA were then intrathecally administered via chronic polyethylene catheters to PNL treated rats. Each compound was administered at a dose of 20 nmol. As shown in figure 5c, the amidated χ-MrIA compounds resulted in a statistically significant ($p<0.01$) increase in mechanical PWT for four hours after intrathecal injection compared to a saline vehicle. Administration of χ-MrIA dendrimers (**6-8**) did not produce any significant differences in PWT in the four hours after injection compared with i.t. injection of a saline vehicle. There was no significant difference in rotarod performance between any of the drugs or controls ($p>0.05$) for up to 4 hours post injection (Figure 5d), which suggests the χ-MrIA dendrimers (**6-8**) and amidated χ-MrIA did not cause any motor impairment/side-effects. A possible explanation of the activity loss in *vivo* is that χ-MrIA dendrimers are unable to diffuse through the spinal cord to reach the NET receptor located in the dorsal horn, the location of the descending inhibitory pathway presumed to be the target of the drug.[23] These results indicate that χ-MrIA dendrimers (**6-8**) possess no pharmacokinetic advantages compared to free χ-MrIA in an intrathecal route of administration.

Conclusion

A comprehensive study of the synthesis, secondary structure and functional activity of χ-MrIA dendrimers with sizes 8 KDa to 22 KDa was undertaken. To assemble each dendrimer, azido modified χ-MrIA with 2 disulfide bonds was successfully attached to alkyne polylysine-based dendrons through optimization of the CuAAc reaction and addition of PEG spacer segment, which led to the formation of homogenous tetramer and octamer dendermeric χ-MrIA constructs. We demonstrated that variation of linker length and dendron size is necessary to obtain full χ-MrIA activity though further optimization was not pursued. NMR structural analysis revealed that multigeneration lysine-dendrimers did not interfere with the structure of the attached χ-MrIA ligands, a key requirement for maintenance of the activity of the structurally sensitive χ-MrIA pharmacophore. The functional *in vitro* activities of the χ-MrIA dendrimers resulted in comparable potency at NET with χ-MrIA-NH_2 and slightly increased potency when compared to azido-PEG-MrIA analogues. In a rat pain study intrathecal *in vivo* delivery of the peptide dendrimer constructs did not lead to pain relief suggesting that χ-MrIA dendrimers of high molecular weight are unable to diffuse through the spinal cord to reach the NET receptor located in the dorsal horn.[23]

Supporting Information. Additional data illustrated the LC-ESI-MS results of azido peptides and alkyne dendrons, the HPLC reaction monitor traces, NMR ^{1}H, ^{13}C and αH chemical shift assignment.

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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; DMSO, dimethyl sulfoxide; TIPS, triisopropylsilane; PEG, polyethylene glycol; CuAAc, copper catalyzed alkyne azide cycloaddition; GPCRs, G-protein coupled receptors; χ-conotoxin MrIA (χ-MrIA); RP-HPLC, reversed-phase high-performance liquid chromatography; LC-MS, liquid chromatography coupled mass spectrometry; NMR, nuclear magnetic resonance; NOESY, nuclear overhauser enhancement spectroscopy; HSQC, Heteronuclear single quantum coherence spectroscopy; TOCSY, total correlated spectroscopy; NET, norepinephrine transporter; NE, norepinephrine; PWT, paw withdrawal threshold; PNL, Partial ligation of the sciatic nerve.

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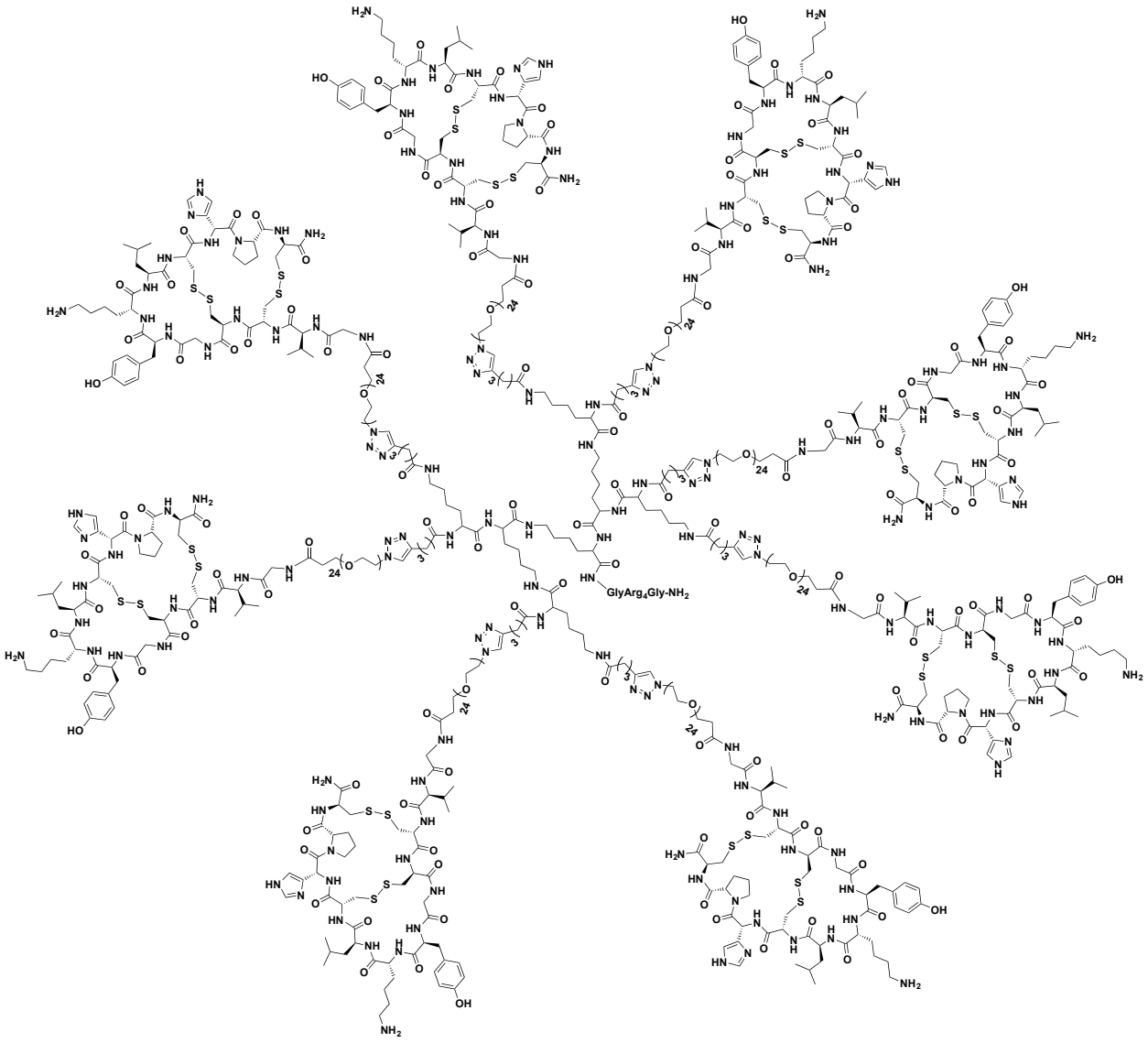

Scheme 1. Schematic view of the dendrimeric peptide system (**7)** which consists of eight disulfide-cyclic χ-MrIA analogues N_3-PEG(24)-CO-[Pro]^{11}MrIA[2-13] (**5)** conjugated to a lysine dendron core of $GlyArg_4Gly$-Lys[Lys]$_2$[Lys]$_4$[CO-$(CH_2)_3$-C≡CH]$_8$ (**3**) by employing copper catalyzed azide-alkyne click chemistry.

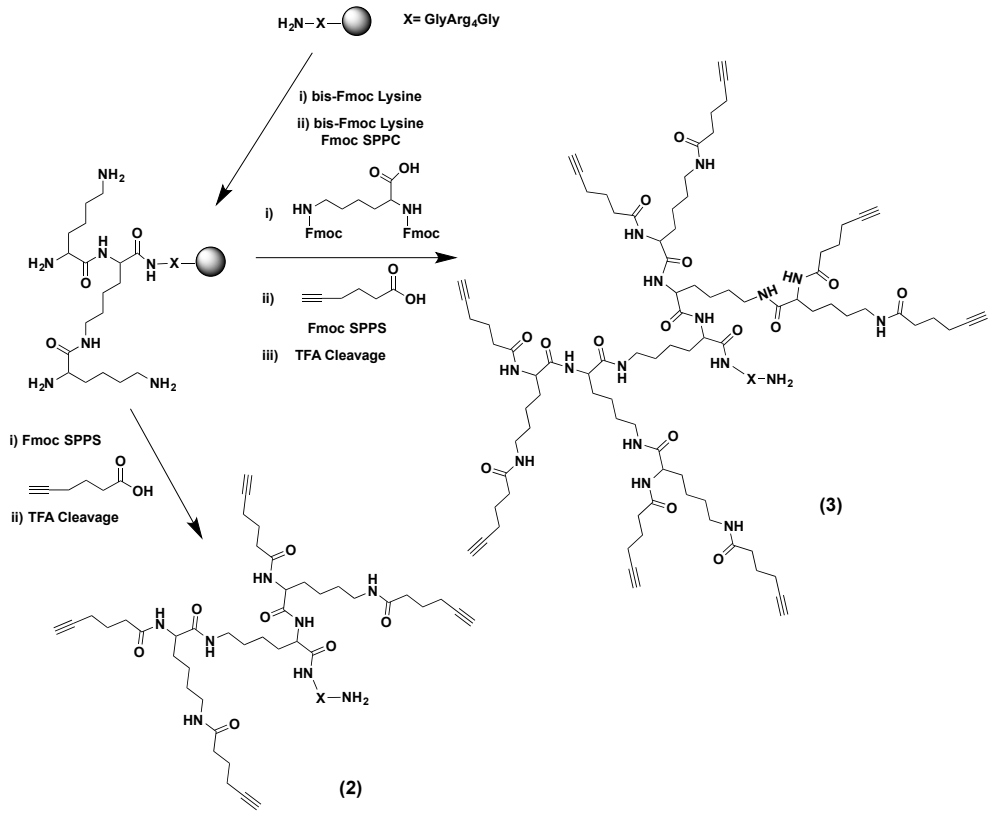

Figure 1. Synthesis route of alkyne modified 4-mer and 8-mer polylysine dendrons (**2** and **3**) employing coupling of 2 and 3 generations respectively of bis-Fmoc-Lysine onto a Gly-Arg$_4$-Gly anchor. Polylysine dendrons were armed for click chemistry by modification with 5-Hexynoic acid.

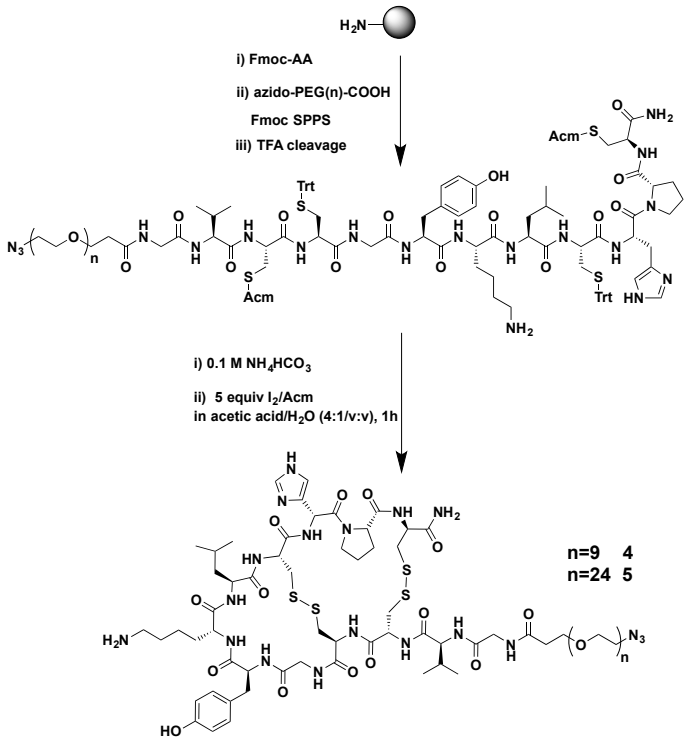

Figure 2. Synthesis route of azido PEG chain modified χ-MrIA analogues (**4** and **5**) employing Fmoc-SPPS with stepwise selective disulfide bond formation using orthogonal Cys(Trt) and Cys(Acm) protection

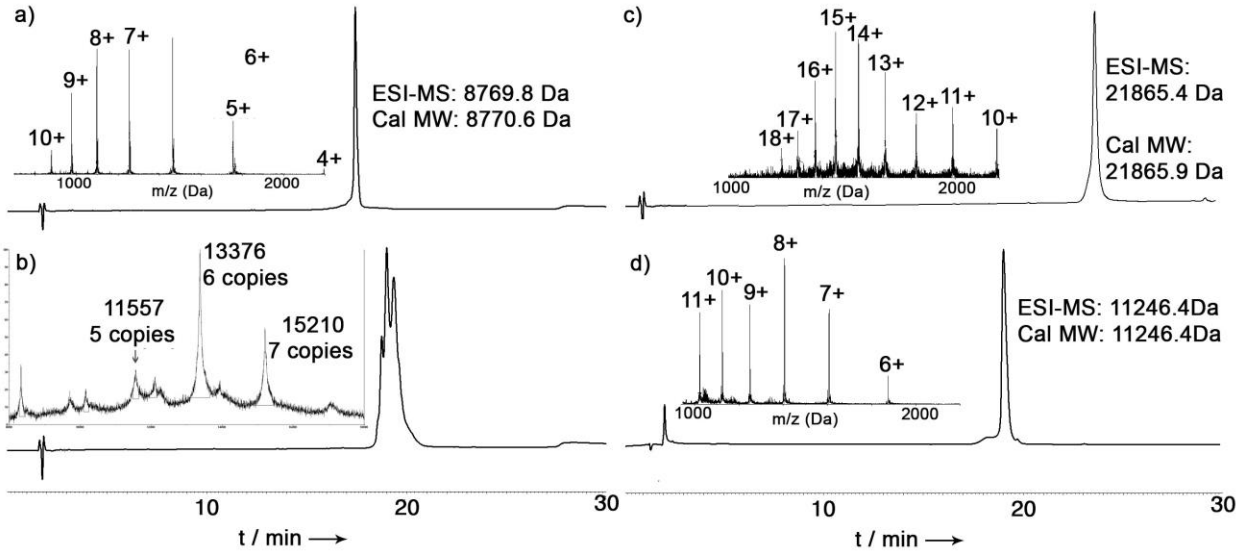

Figure 3. LC-MS results for the synthesis of a) 4xMrIA-PEG(9)-D **(6)**, b) χ-MrIA dendrimer mixture obtained from click reaction of **3** and **4** showing insufficient decoration with 5, 6, and 7 attached χ-MrIA units, c) 8xMrIA-PEG(24)-D (**7**) and d) 4xMrIA-PEG(24)-D (**8**).

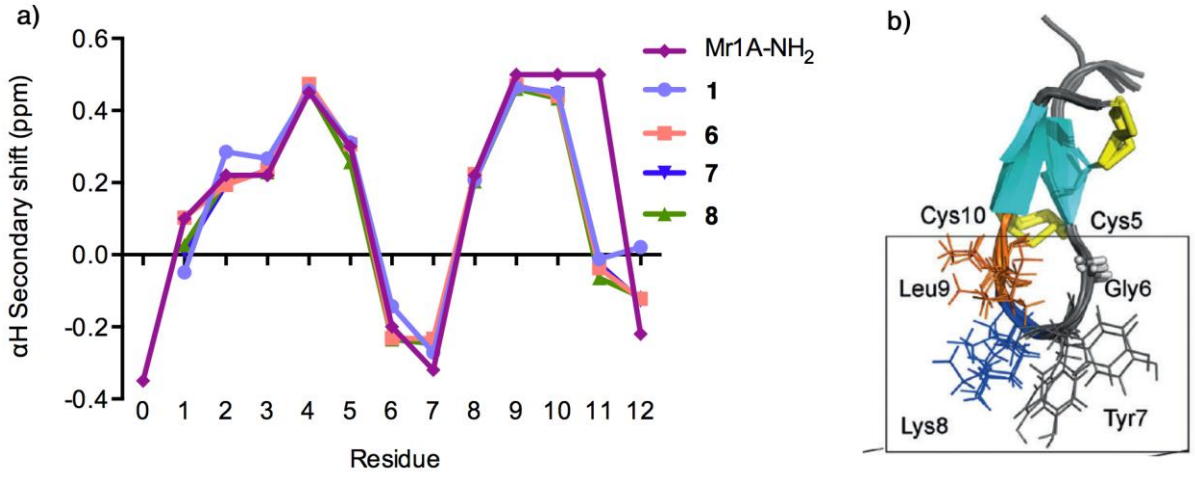

Figure 4. a) Secondary α-shifts of χ-MrIA-NH_2,[31] [Pro][11]MrIA-NH_2[2-13] (**1**) and χ-MrIA dendrimers (**6-8**). The secondary shifts were determined by subtracting the random coil shift[50] from the αH shift; b) NMR structure of native χ-MrIA highlighting the pharmacophore located within residues Gly^6, Tyr^7, Lys^8 and Leu^9 of this class of norepinephrine reuptake inhibitor.[20] Secondary α-shifts confirmed the structural integrity of the attached peptide molecules and pharmacophore region Gly^6 to Leu^9.

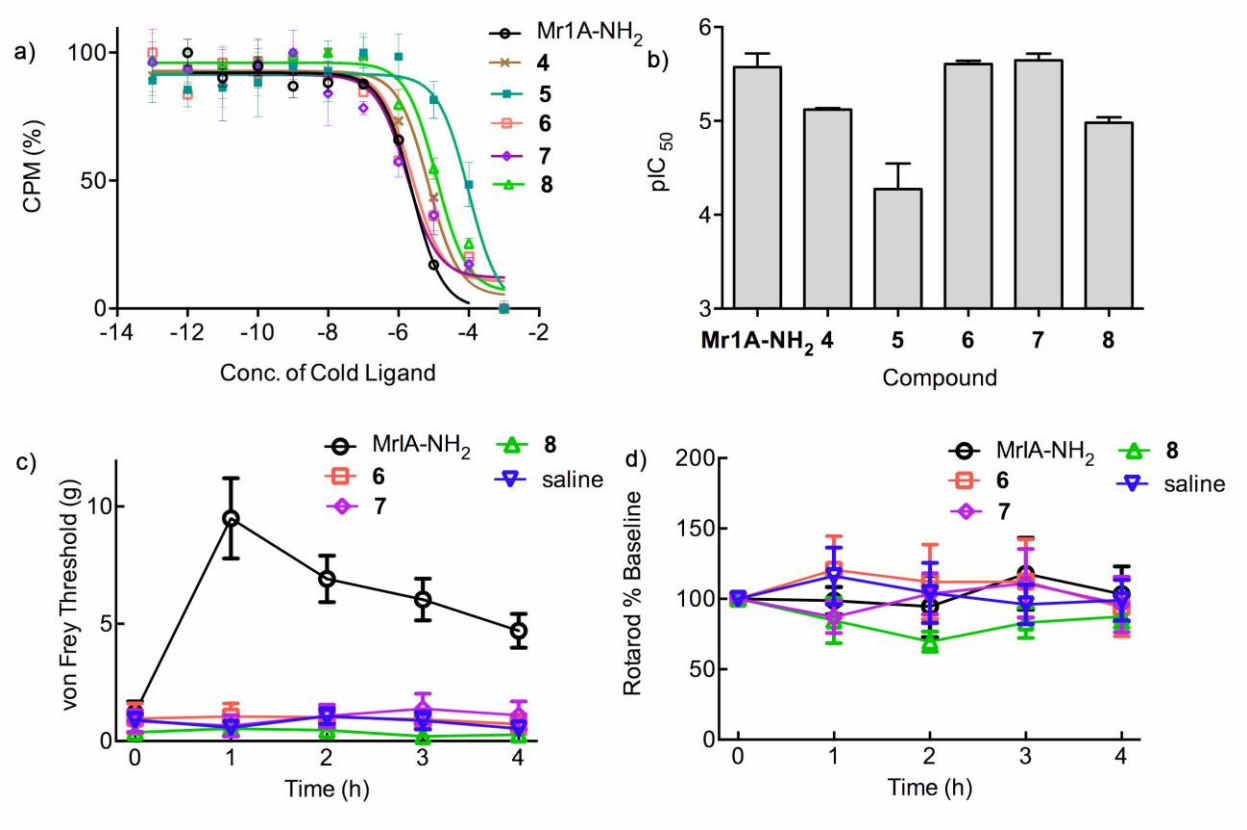

Figure 5. a) Functional norepinephrine reuptake assay of the χ-MrIA dendrimers (**6-8**), monomer azide analogues (**4** and **5**) and χ-MrIA-NH_2. Representative concentration-response curves performed in triplicate for each sample; b) comparison of pIC_{50} values of χ-MrIA dendrimers (**6-8**), monomer analogue (**4** and **5**) and χ-MrIA-NH_2 obtained from NE uptake inhibition assay. Data are presented as mean ± SEM of n = 3 replicates and are representative of 3 independent experiments; c) *in vivo* rat pain model: the mean (±SEM) von Frey threshold versus time curves for the relief of mechanical allodynia produced by i.t. administration of 20 nmol of each χ-MrIA dendrimer (**6-8**), amidated χ-MrIA as a positive control and 0.9% saline as a negative/vehicle control in PNL treated rats; d) Central effect assessment: rotarod performance of the χ-MrIA dendrimers (**6-8**), amidated χ-MrIA and 0.9% saline in PNL treated rats. 4xMrIA-PEG(9)-D n = 5, 4xMrIA-PEG(24)-D n = 5, 8xMrIA-PEG(24)-D n = 6, χ-MrIA-NH_2 (n=8) and 0.9% saline (n=7).

Table 1. ESI-MS analysis of **2**-**8** and norepinephrine reuptake inhibition (IC_{50}) by azido-PEG-[Pro]^{11}MrIA[2-13]-NH_2 (**4** and **5**), χ-MrIA dendrimers (**6**-**8**) and χ-MrIA-NH_2 (1–4, 2–3 disulfide bond). IC_{50} values are means ± SEM of 3 separate experiments (performed in triplicate).

No.	Analogue	Calculated MW (Da)	Observed MW (Da)	IC_{50} (μ M)
	χ -MrIA-NH_2			3.0 ± 0.5
				2.45 ± 0.07[a]
2	4-mer alkyne dendron	1516.6	1516.4	-
3	8-mer alkyne dendron	2405.9	2406.4	-
4	N_3-PEG(9)-[Pro]^{11}MrIA[2-13]-NH_2	1813.5	1813.0	7.8 ± 0.5
5	N_3-PEG(24)-[Pro]^{11}MrIA[2-13]-NH_2	2432.5	2432.0	96.5 ± 1.5
6	4xMrIA-PEG(9)-D	8770.6	879.8	2.5 ± 0.2
7	8xMrIA-PEG(24)-D	21865.9	21865.4	2.3 ± 0.5
8	4xMrIA-PEG(24)-D	11246.4	11246.4	12 ± 0.09

[a] IC_{50} value of χ-MrIA-NH_2 measured in literature[29].