

Structure-function relationships of tachykinin peptides from octopus venoms

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Abstract

Introduction

Tachykinins are a highly evolutionarily conserved group of peptides found in invertebrate and vertebrate species, functioning as neurotransmitters and neuromodulators of both [the](#) central and peripheral nervous systems (Van Loy *et al.*, 2010). [The mammalian tachykinins, neurokinin A, neurokinin B and Substance P are sensory neuropeptides involved in both nociception and inflammation. Aside from afferent functions, the mammalian tachykinins also exhibit efferent functions and](#) participate in the regulation of [several](#) physiological processes [including smooth muscle contractility in a variety of tissues](#) (Riberio, 1991, [Khawaja & Rogers, 1996](#)).

The actions of tachykinin peptides are mediated by one or more tachykinin receptors. Three subtypes of vertebrate tachykinin receptors, known as neurokinin receptor 1 (NK1R), neurokinin receptor 2 (NK2R) and neurokinin receptor 3 (NK3R) and numerous subtypes of invertebrate tachykinin receptors have been described to date (Maggi, 1995; Van Loy *et al.*, 2010). Neurokinin receptors have been shown to act via G_q/11 coupling proteins, increasing inositol phosphate 3 and diacylglycerol (DAG) levels within the cells binding agonist (Pinnock *et al.*, 1994). To date, a number of characteristic tachykinin amino acid motifs have been found to be crucial to the structure-activity relationships of tachykinins and tachykinin-like peptides, such as the common FXGLM-amide motif found in vertebrate tachykinins such as Substance P, neurokinin A and neurokinin B (Van Loy *et al.*, 2010). Invertebrate tachykinins commonly share the C-terminal motif FXGXR-amide motif, which has not been identified in vertebrate tachykinins.

Among the invertebrate tachykinins identified thus far, four weaponised tachykinin-like peptides have been isolated from octopus venom: octopus tachykinins 1 and 2 from *Octopus vulgaris* (Oct-TK-I and Oct-TK-II) (Kanda *et al.*, 2004); Oct-TK-III from *Octopus kaurna* (Fry, 2009); and eledoisin from *Eledone cirrhosa* (Anastasi & Erspammer, 1962). While octopus species feed on both vertebrate and invertebrate prey (Sazima & Bastos-de-Almeida, 2006), one might predict a combination of venom elements effective on each target type, with evolutionary conservation leading to toxins with high efficacy on both vertebrate and invertebrate receptors, yet only vertebrate type tachykinins have thus far been identified in octopus venom and only Oct-TK-I and Oct-TK-II have been tested for vertebrate activity (Kanda *et al.*, 2004). Thus, the over-arching [aim](#) of this study was to compare the differential effects of octopus venom tachykinin peptides upon invertebrate and vertebrate tissue preparations.

Materials and Methods

Peptide synthesis & purification:

In order to explore potential neofunctionalisation derivations, we constructed Oct-TK-I (KPPSSSEFIGLM-amide), Oct-TK-II (KPPSSSEFVGLM-amide) and Oct-TK-III (DPPSDDEFVSLM-amide). Protected Fmoc-amino acid derivatives were purchased from Novabiochem (address) or Aussep (Melbourne, Australia). The following side chain protected amino acids were used: His(Trt), Hyp(tBu), Tyr(tBu), Lys(Boc), Trp(Boc), Arg(Pbf), Asn(Trt), Asp(OtBu), Glu(OtBu), Gln(Trt), Ser(tBu), Thr(tBu), Tyr(tBu). All other Fmoc amino acids were unprotected. Peptide-synthesis grade dimethylformamide (DMF), dichloromethane (DCM), diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were supplied by Aussep. 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), triisopropyl silane (TIPS), HPLC grade acetonitrile, acetic anhydride and methanol were supplied by Sigma Aldrich (address). The resin used was Fmoc-Arg(Pbf)-wang resin (0.33 mmol/g) from Peptide International. Ethane dithiol (EDT) was from Merck.

Peptides were synthesised on a Protein Technology (Symphony) automated peptide synthesizer using Fmoc-Arg(Pbf)-Wang resin (0.1 mmol). Assembly of the peptides was performed using HBTU/DIEA in-situ activation protocols (Schnolzer *et al.*, 2007) to couple the Fmoc-protected amino acid to the resin (5 equiv. excess, coupling time 20 min). Fmoc deprotection was performed with 30% piperidine/DMF for 1 min followed by a 2 min repeat. Washes were performed 10 times after each coupling as well as after each deprotection step. After chain assembly and final Fmoc deprotection the peptide resins were washed with methanol and dichloromethane and dried in a stream of nitrogen. Cleavage of peptide from the resin was performed at room temperature (RT) in TFA:H₂O:TIPS:EDT (87.5:5:5:2.5) for 3 h. Cold diethyl ether (30 mL) was then added to the filtered cleavage mixture and the peptide precipitated. The precipitate was collected by centrifugation and subsequently washed with further cold diethyl ether to remove scavengers. The final product was dissolved in 50% acetonitrile and lyophilized to yield a white solid product. The crude peptide was examined by reversed-phase HPLC for purity and the correct molecular weight confirmed by electrospray mass spectrometry (ESMS).

Analytical HPLC runs were performed using a Shimadzu HPLC system LC10A with a dual wavelength UV detector set at 214 nm and 254 nm. A reversed-phase C₁₈ column (Zorbax 300-SB C-18; 4.6 x 50 mm) with a flow rate of 2 mL/min was used. Elution was performed using a 0–80% gradient of Buffer B (0.043% TFA in 90% acetonitrile) in Buffer A (0.05% TFA in water) over 20 min. Crude peptides were purified by semi-preparative HPLC on a Shimadzu HPLC system LC8A with a reversed-phase C₁₈ column (Vydac C-18, 25 cm x 10 mm) running at a flow rate of 5 mL/min with a 1%/min gradient of 5–50% Buffer B. The purity of the final product was evaluated by analytical HPLC (Zorbax 300SB C18: 4.6 x 100 mm) with a flow rate of 1 mL/min and a 1.67 %/min gradient of Buffer B (5–45%). The final purity of all synthesized peptides was >95%. Electrospray mass spectra were collected inline during analytical HPLC runs on an Applied Biosystems API-150 spectrometer operating in the positive ion mode with an OR of 20, Rng of 220 and Turbospray of 350 degrees. Masses between 300 and 2200 amu were detected (Step 0.2 amu, Dwell 0.3 ms).

CD spectrometry:

Far UV length spectrometric data was recorded from 250 nm to 190 nm on Jasco j-810 polarimeter (Jasco, Tokyo, Japan). The instrument was calibrated using double distilled water (DDW). A cell with a capacity of 400 ul and a path length of 0.1 cm was used. All experiments were carried out at room temperature. The following parameters were used: Step resolution 0.5 nm, scan speed 20 nm/min. Each was obtained from an average of 5 scans. The sample concentration was 333 uM in DDW and 1 mM in DDW. Structural calculations were undertaken using the Dichroweb webservice provided by The University of London, Department of Crystallography using the K2D matrix (Whitmore & Wallace, 2004; 2008).

Bioactivity testing using rat ileum:

Male rats aged 11-16 weeks were [euthanased](#) using CO₂ and cervical [dislocation](#), with ileum dissected, cleaned, and cut into 1 cm [segments](#), placed in Tyrode's buffer (20 L DDW, 160.1 g NaCl, 3.4 g KCl, 1.14 g H₂PO₄, 4.27 g MgCl₂ 6H₂O, 20 g NaHCO₃, 19.8 g glucose, 4.0 g ascorbic acid, 36 ml 1M CaCl₂), aerated with [carbogen](#) gas. Sections were threaded and attached to a W-hook for placement in the organ bath apparatus. Baseline tension between 10-20 mN was applied via tension transducer, and tissues were allowed to equilibrate to experimental conditions for a minimum of 30 minutes, while recording baseline contractile behavior. Test peptide was then applied to tissue (1 nM – 3 [µM](#)), recording responses using Lab Chart, followed by analysis

of data using GraphPad Prism. One peptide was tested per tissue section with n=9 tissues used for each peptide.

Bioactivity testing using crayfish hindgut:

Spontaneous contractions were recorded from isolated crayfish hindguts according to procedures published elsewhere (Mercier et al., 1997; Wrong et al., 2003). Hindguts were dissected from male *Procambarus clarkii* with carapace lengths of 2-5 cm that had been ~~ethanized~~ ethanased following cold anaesthesia. The hindguts were placed in crayfish physiological saline (van Harreveld, 1936) containing 205 mM NaCl, 5.3 mM KCl, 13.5 mM CaCl₂, 2.45 mM MgCl₂ and 5 mM HEPES (pH 7.4). One end of each hindgut was pinned to the bottom of the dish, and the other was connected to a Grass FT03 force-displacement transducer. Signals were amplified using a Grass MOD CP122A amplifier and were acquired and analysed on a PC-compatible computer using a custom-built, computerized data acquisition system and software (Technical Services Division, Brock University, St. Catharines, ON, Canada). Solutions were applied directly to the hindgut using a pipette. Baseline recordings were taken for at least five minutes prior to exchanging saline for experimental solutions.

Results and Discussion

Our findings indicate that OCT-TK-I, OCT-TK-II and OCT-TK-III are differentially active when assayed for invertebrate- and vertebrate-specific effects. In the isolated rat ileum tissue assay, all three peptides showed classic tachykinin responses, in that after addition to tissue there was an initial decrease in contractility relative to baseline contractions, followed by an increase to peak contraction dependent upon concentration, and then a period of oscillation until the peptide was washed from the organ bath (Figures 1 and 2). When tested on the crayfish hindgut, all three peptides elicited increases in contraction (Figure 3), with similar EC₅₀ values (Table 1), although the EC₅₀ are lower for Oct-TK-III. The increase in contraction amplitude was statistically indistinguishable between the three peptides, indicating a high similarity in efficacy for their inotropic effect. There were differences, however, in effects on contraction frequency (Figure 3A). Both Oct-TK-II and Oct-TK-III elicited concentration-dependent increases in sponateous contraction frequency. However contractions in the presence of Oct-TK-III were approximately twice as frequent at each concentration tested. Thus, Oct-TK-III is approximately twice as efficacious as Oct-TK-II at increasing contraction frequency. Oct-TK-I did not elicit a significant change in contraction frequency at any of the concentrations tested. Frequency data for 5 x 10⁻⁷ M Oct-TK-I were not significantly different from those of Oct-TK-III and Oct-TK-II at 5 x 10⁻⁹ M, which were below the threshold for chronotropic effects. EC₅₀ values for chronotropic effects of Oct-TK-III and Oct-TK-II, which were estimated based on the assumption that both peptides achieved saturation at 5 x 10⁻⁷ M, do not reflect the difference in potency. Overall, the results indicate that the three tachykinins elicit inotropic effects that are nearly indistinguishable, but their chronotropic effects reveal differences in efficacy or possibly potency, with a relative selectivity order of Oct-TK-III > Oct-TK-II > Oct-TK-I.

The lack of structural integrity of tachykinin peptides is supported by our circular dichroism spectrometric analysis (Figure 4), which showed the tachykinin peptides examined as being principally unstructured, with the exception of OCT-TK-III which shows a spectrum indicative of beta sheet content and random coil content. All other spectra are indicative of unstructured peptides and yielded significant error during structural determination via Dichroweb (Whitmore & Wallace, 2004; 2008). This may indicate that neurokinin receptors play a role in stabilizing the otherwise randomly coiled tachykinin peptides. In addition to being the most structured form, OCT-TK-III was also distinguished from OCT-TK-I and OCT-TK-II in having a N-terminal negatively charged residue (D), while the latter two both had a positively charged residue (K) at this location. As order of potency for effects on the rat ileum (OCT-TK-III>OCT-TK-II ≈ OCT-TK-I) were in reasonable accord with the relative order effects on crayfish hindgut, the octopus tachykinins act on vertebrate and invertebrate receptors that appear to be substantially similar. These observations generally support earlier suggestions (Kanda et al., 2004) that evolutionary conservation has led to the appearance of peptides with similarly high efficacy on vertebrate and invertebrate receptors,

We do not know which receptors mediate the effects of OCT-TK-I, II and II on crayfish hindgut. All three peptides contain the carboxy terminal sequence S/GLM-amide, but crustacean tachykinins typically contain the sequence GMR-amide (Cansela et al. 2012; Christie et al. 1997; Dircksen et al. 2011; Hui et al. 2011; Jiang et al. 2012). Cockroach hindgut contracts in response to peptides (e.g. Substance P) containing the carboxyl terminal sequence GLM-amide with

[thresholds around \$10^{-7}\$ - \$10^{-5}\$ M, but substituting Arg for Met at the C-terminal increases potency 100-fold \(Ikeda et al., 1999\). Five invertebrate tachykinin receptors have been reported \(Van Loy, 2010\), including three from arthropods that show a marked preference for the C-terminal GLR-amide over GLM-amide \(Birse et al., 2006; Johnson et al. 2003; Poels et al. 2007, 2009; Torfs et al. 2000, 2002\). An early report, however, showed that the *Drosophila* tachykinin receptor DTKR responds to micromolar concentrations of Substance P when expressed in *Xenopus* oocytes \(Li et al., 1991\). Thus, the responses we report might be mediated by a receptor related to DTKR. We cannot rule out the possibility that arthropods might contain receptors that are selective for peptides with the C-terminal sequence GLM-amide. The mosquito, *Aedes aegypti*, contains two peptides \(Sialokinin I and II\) with GLM-amide at the C-terminal, but both are present in the salivary gland and are thought to act on mammalian tachykinin receptors to cause vasodilation \(Champagne et al. 1994\).](#)

As venoms are typically combinations of compounds with high target receptor specificity and potency, venoms are a natural source for novel parent compounds of potential medicinal benefit. In this study, we examined the differential effects of the octopus venom peptides Oct-TK-I, Oct-TK-II and Oct-TK-III for vertebrate and invertebrate tissue-specific contractile activity using the rat ileum (*Rattus norvegicus*) and crayfish hindgut (*Procambarus clarkia*) assays. These results show that the three versions of tachykinin operate differentially but with the effects consistent between the invertebrate and vertebrate forms. However, it is not clear that any of the present results are physiologically related to predation. Further work with other venom components or with other physiological targets could investigate mechanisms of predation more directly. It is hoped that this work increases our working knowledge of these peptides, their structure-activity features, and their identification of possible receptor sites of action.

[Tachykinins are known to play important roles in various physiological processes and systems in humans. These include peripheral sensory mechanisms such as nociception and inflammation as well as autonomic functions such as smooth muscle contractility in the vascular, gastrointestinal and genitourinary systems \(Khawaja & Rogers, 1996\). In addition, tachykinins are involved in central nervous system pathways mediating pain, anxiety, motor co-ordination and cognition \(Khawaja & Rogers, 1996\). Therefore, venom tachykinins may provide novel insights into the development of potent and selective tachykinin receptor ligands which could have potential benefits in the treatment of a variety of disorders including irritable bowel syndrome, lower urinary tract symptoms, asthma, chronic pain, depression, Parkinson's disease and Alzheimer's disease.](#)

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TABLE 1. Summary of peptides, amino acid sequences, and relative potency.

| Designation | Amino Acid Sequence | Rat Ileum EC₅₀ (M) | Crayfish Hindgut EC₅₀ (M) Frequency | Crayfish Hindgut EC₅₀ (M) Amplitude |
|--------------------|----------------------------|--|---|---|
| OCT-TK-I | KPPSSSEFIGLM-amide | 5.55 x 10 ⁻⁸ | - | 4.4 x 10 ⁻⁸ |
| OCT-TK-II | KPPSSSEFVGLM-amide | 4.37 x 10 ⁻⁷ | 4.9 x 10 ⁻⁸ | 4.2 x 10 ⁻⁸ |
| OCT-TK-III | DPPSDDEFVSLM-amide | 2.28 x 10 ⁻⁷ | 4.4 x 10 ⁻⁸ | 1.8 x 10 ⁻⁸ |

Figure 1: Representative recording of tachykinin effect upon contractile activity of rat ileum smooth muscle at 100 nM concentration. The x-axis shows the time in minutes, seconds, and partial seconds after the start of recording, while the y-axis has divisions of 2.5 mN of tension. Note the initial decrease in tension observed immediately after drug addition, followed by contraction, and the sustained pattern of contraction

Figure 2: Mean concentration-response curve of rat ileum smooth muscle preparation showing SEM and n=9: A) Tachykinin-Oct-1 EC₅₀ concentration was estimated to be 2.28×10^{-7} M; B) Tachykinin-Oct-2 EC₅₀ concentration was estimated to be 4.37×10^{-7} M; Tachykinin-Oct-3 EC₅₀ concentration was estimated to be 5.55×10^{-8} M.

Figure 3: Mean magnitude OCT-TK-I, OCT-TK-II and OCT-TK-III induced crayfish hindgut contractions A) frequency and B) amplitude. *-indicates P<0.05.

Figure 4: A) Comparative circular dichroism of Oct-TK-I, Oct-TK-II and Oct-TK-III at 333 μ M. While Oct-TK-I and Oct-TK-II are principally unstructured in purified water, Oct-TK-III displays local minima at 222 and 198 nm, respectively. Structure predictions from Dicroweb using K2D matrix indicates approximate β -sheet content (35%), alpha helix (9%) and random coiling (56%), with an error of 18.2%. B) Oct-TK-III concentration-dependent helicity.

Figure 1

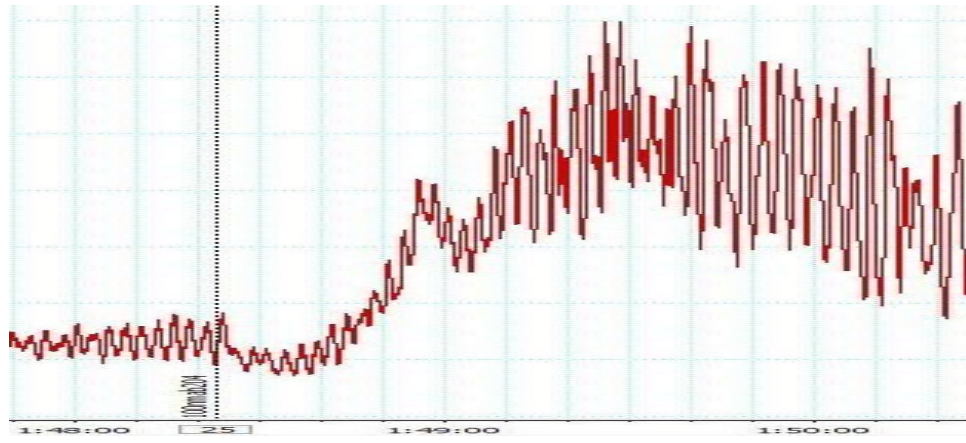
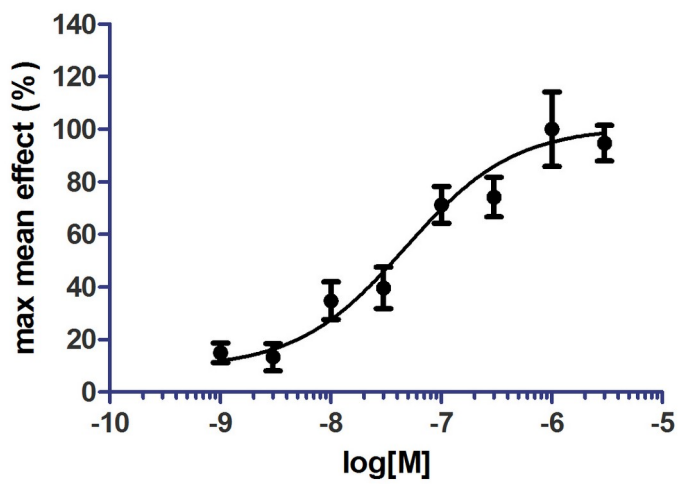
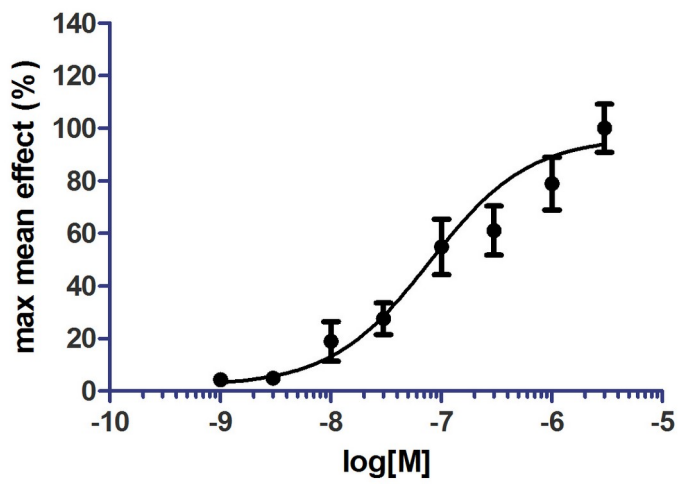


Figure 2

A)



B)



C)

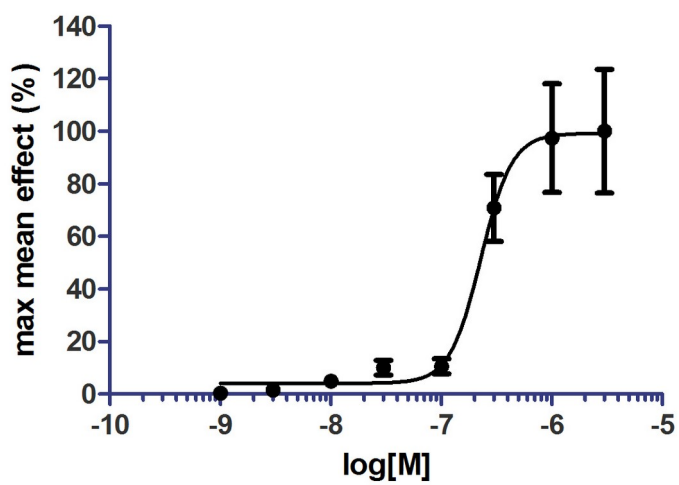


Figure 3

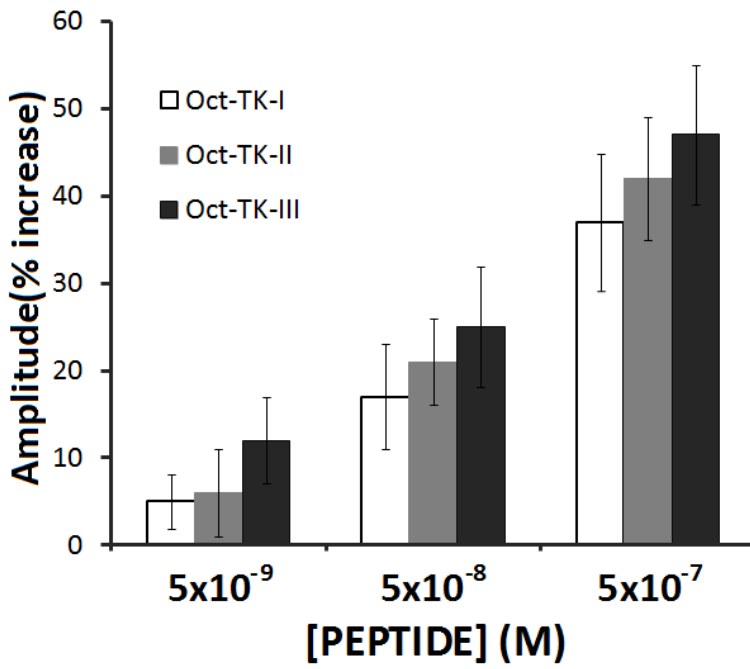
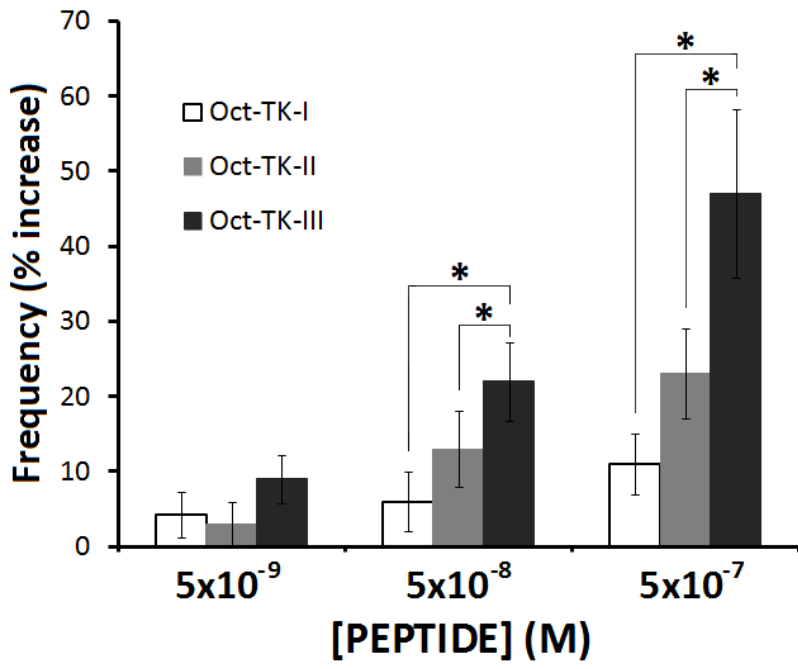
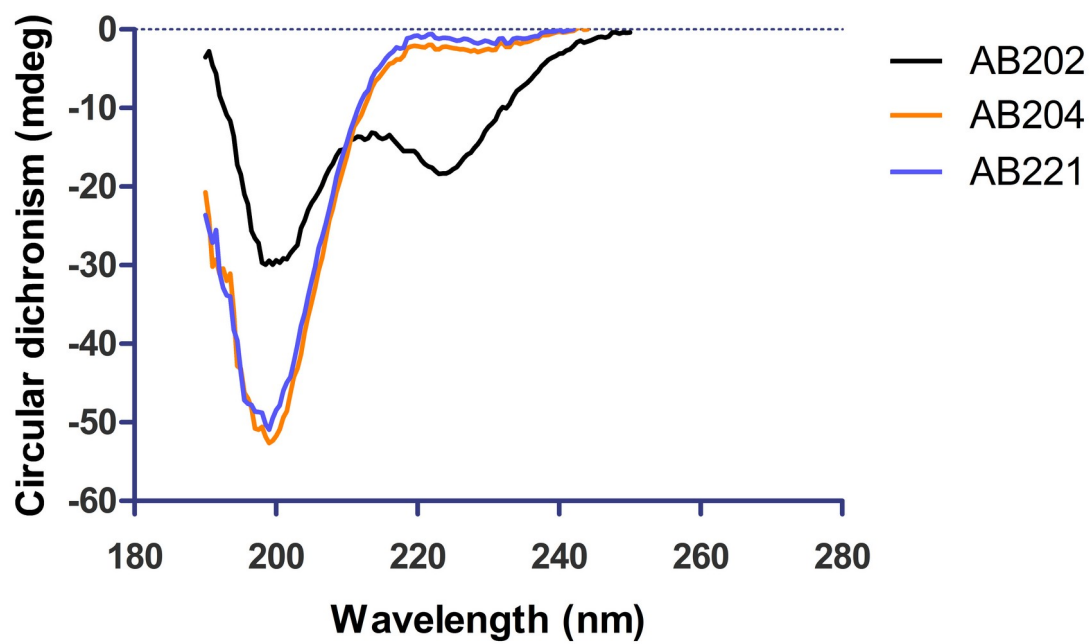


Figure 4

A)



B)

