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ARG-13 OF B-TYPE NATRIURETIC PEPTIDE RECIPROCALLY MODULATES BINDING TO GUANYLYL CYCLASE BUT NOT CLEARANCE RECEPTORS[#]

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Running title: BNP Bifunctional Peptides

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Introduction: 448
Discussion: 509

Abbreviations

ANP: atrial natriuretic peptide; BNP: B-type natriuretic peptide; CNP: C-type natriuretic peptide; GC-A: guanylyl cyclase-A; GC-B: guanylyl cyclase-B; NPR-C: natriuretic peptide receptor C or natriuretic peptide clearance receptor

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ABSTRACT

B-type natriuretic peptide (BNP) decreases cardiac preload and hypertrophy. As such, synthetic BNP, nesiritide, was approved for the treatment of acutely decompensated heart failure. However, two problems limit its therapeutic potential. First, ensuing hypertension decreases urine output, and secondly, guanylyl cyclase-A (GC-A), the primary signaling receptor for BNP, is downregulated in heart failure. Thus, alternative or chimeric natriuretic peptides maintaining the renal but lacking the vasorelaxation properties of BNP provide an alternative approach. Here, we examined the ability of single amino acid substitutions in the conserved 17-amino acid disulfide ring structure of human BNP to activate GC-A and guanylyl cyclase-B (GC-B), which is not reduced in heart failure. We hypothesized that substitution of highly conserved residues in BNP with highly conserved residues from a GC-B specific peptide would yield BNP variants with increased and decreased potency for human GC-B and GC-A, respectively. Substitution of Leu for Arg13 (L-BNP) yielded a five-fold more potent activator of GC-B and seven-fold less potent activator of GC-A compared to wild-type. L-BNP also bound GC-A 4.5-fold less tightly than wild-type. In contrast, substitution of Met for Ser 21 (M-BNP) had no effect. A peptide containing both the Leu and Met substitutions behaved similarly to L-BNP. Meanwhile, wild-type and L-BNP bound the natriuretic peptide clearance receptor with similar affinities. These data indicate that Arg-13 of BNP is a critical discriminator of binding to guanylyl-cyclase-linked but not clearance natriuretic peptide receptors supporting designer natriuretic peptides as an alternative to wild-type BNP for the treatment of heart failure.

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INTRODUCTION

Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are released from the heart in response to increased cardiac volume (Potter et al., 2009). Acutely, they increase renal fluid secretion and arterial vasodilation. Chronically, they inhibit cardiac hypertrophy. The signaling receptor for ANP and BNP is guanylyl cyclase-A (GC-A), also known as natriuretic peptide receptor-A. C-type natriuretic peptide (CNP) activation of guanylyl cyclase-B (GC-B), also known as natriuretic peptide receptor-B, preferentially dilates veins as opposed to arteries (Wei et al., 1993), and like GC-A, inhibits cardiac hypertrophy (Langenickel et al., 2006). Both GC-A and GC-B are transmembrane guanylyl cyclases that catalyze the synthesis of cGMP upon ligand binding. The third natriuretic peptide receptor, natriuretic peptide receptor C (NPR-C), binds all three natriuretic peptides with similar affinity and clears them from the circulation through receptor-mediated endocytosis degradation.

BNP has been extensively studied as a potential therapeutic for the treatment of congestive heart failure and renal diseases (Boerrigter et al., 2009). The U.S. Food and Drug Administration approved recombinant BNP (nesiritide) for the treatment of acutely decompensated heart failure in 2001. However, subsequent reports indicated that hypotension-dependent reductions in renal perfusion pressures ultimately decrease renal fluid loss, which limits the use of nesiritide in the treatment of heart failure (Sackner-Bernstein et al., 2005). Thus, natriuretic peptides with reduced hypotensive actions but maintained renal actions may be superior to nesiritide.

One approach to producing more therapeutic natriuretic peptides is to create chimeric molecules that activate both guanylyl cyclase receptors. GC-A mediates the renal affects of ANP and

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BNP(Kishimoto et al., 1996), whereas GC-B mediates the venodilation effect of CNP but does not activate the kidney. Additionally, unlike GC-A, cardiac GC-B is not downregulated in response to congestive heart failure (Dickey et al., 2007). Thus, our rationale was to produce peptides with reduced affinity for GC-A but increased affinity for GC-B in order to decrease cardiac preload and hypertrophy observed in heart failure. BNP was used as the starting backbone for the chimeric peptides due to its increased half-life (~20 min vs. 1-2 min for ANP and CNP). Residues in the 17-amino acid disulfide ring of BNP were substituted in order to modulate receptor-binding affinity (Fig. 1). Eleven of the 17 residues are identical between human ANP, BNP and CNP, which leaves only two unique triad sequences as potential regulators of binding specificity (Fig. 1). Here, we describe the effects of two single amino acid substitutions -individually or in combination - on the ability of BNP to activate human GC-A and GC-B or bind NPR-C. We found that a single substitution in the first divergent triad region markedly and reciprocally modulates GC-A and GC-B activation while substitutions in the second triad were ineffective.

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MATERIALS AND METHODS

Peptides and reagents. Anaspec (Fremont, CA) synthesized wild-type and BNP derivative peptides. The lyophilized peptides were reconstituted in deionized water, aliquoted and stored at -80°C until use. Cyclic GMP radioimmunoassay kits were from Perkin Elmer (Boston, MA). ^{125}I-ANP was from Phoenix Pharmaceuticals.

Cells. Human embryonic kidney 293 cells stably expressing human GC-A or GC-B were cultured as previously described (Dickey et al., 2008).

Whole cell cGMP assays. Cells plated in 48-well plates were incubated in serum-free medium for 4 hrs. The cells were pretreated for 10 min at 37°C in DMEM containing 25mM HEPES, pH 7.4 and 1mM 1-methyl-3-isobutylxanthine (IBMX) after aspiration of the starvation medium. After pretreatment, the medium was replaced with the same medium containing various concentrations of natriuretic peptides. The cells were stimulated for 3 min and then the assay was terminated by aspiration and the addition of 0.2 ml ice-cold 80% ethanol. Cyclic GMP concentrations were estimated by radioimmunoassay as previously described (Dickey et al., 2009).

Membrane guanylyl cyclase assays. Crude membranes were prepared as previously described (Bryan et al., 2007). Twenty µl membranes were assayed for 3 min at 37°C containing 1 mM Mg-GTP in the presence or absence of various concentrations of natriuretic peptide as previously described (Dickey et al., 2009). The assayed were stopped with the addition of 0.4 ml cold 50

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mM sodium acetate buffer containing 5 mM EDTA and placed on ice. An aliquot was assayed for cGMP using a cGMP radioimmunoassay kit from Perkin Elmer.

Whole cell binding assays. Cells were plated on 24-well plates precoated with polylysine. When the cells were 75-90% confluent, the growth media was replaced with 0.2% BSA in DMEM for 1-2 h. Binding media containing 1% BSA and ^{125}I-ANP was prepared on ice. Binding media containing increasing concentrations of unlabelled ligand were added to the cells and incubated at 4°C for 1 h. The cells were washed with ice-cold PBS to remove non-specific bound tracer. 0.5 ml of 1N NaOH was added to solubilize the cells. The cell extracts were counted in a Beckman gamma 5500 counter.

Statistics. The whole cell experiment was performed in triplicate in three separate assays. Concentrations required to achieve 50% of the maximum effect (EC_{50}) were calculated with Prism software using a Sigmoidal dose response curve fit. Differences between treatments was determined by paired t test where $p < 0.05$ was considered significant. The graphs of the guanylyl cyclase assays were from two separate assays of two individual samples assayed in duplicate. The binding experiment graphs represent three (GC-A) or two (NPR-C) separate experiments assayed in triplicate. Dissociation constants for ^{125}I-ANP binding were calculated using Prism software using a one-site competition model. Differences between treatments were determined by paired t test (GC-A) or an F test (NPR-C) where $p < 0.05$ was considered significant.

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RESULTS

The goal of this study was to create derivatives of BNP with reduced potency for GC-A and improved potency for GC-B. All natriuretic peptides contain an amino-terminal region followed by a 17-amino acid disulfide ring (Fig. 1). Of the 17 residues within the ring, eleven are identical (shaded residues in Fig. 1). Since CNP binds a different receptor than ANP or BNP, identical residues between CNP or ANP and BNP cannot determine binding specificity. Previous studies from our group suggested that triple substitutions in one of the two variable regions in the 17-amino acid ring of a C-terminally extended form of CNP called CD-NP, modified the binding preference for GC-A and GC-B (Dickey et al., 2008). Here, we investigated the effect of single or double substitutions on the ability of BNP to activate GC-A and GC-B.

The first unique triad consists of RKM and LKL in BNP and CNP, respectively. Since the middle K is conserved in both peptides, we mutated the first amino acid of this triad from an R to an L in BNP and called it L-BNP. The second triad sequence is SSS in BNP and GSM in CNP, respectively. The first residue (G) in the second CNP triplet is unlikely to cause any changes in receptor affinity since it is also a G in ANP, another GC-A agonist. The second residue in the triplet is an S, which is identical between BNP and CNP. Therefore, the third amino acid in the first BNP triplet was the sole focus for substitution analysis of this region because it is the only one that significantly varies between peptides that activate either GC-A or GC-B. We substituted the third S in BNP with an M from CNP to make M-BNP. A derivative peptide containing both amino acid substitutions called LM-BNP was also examined.

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Whole cell stimulation of human GC-A. The ability of the BNP derivative peptides to elevate cGMP concentrations in intact 293 cells stably expressing GC-A was examined. These cells do not express endogenous GC-A or GC-B so natriuretic peptide-dependent cGMP elevations can be solely ascribed to the stably transfected receptor. Cells were incubated with increasing concentrations of the wild type or mutant peptides for three minutes and then intracellular cGMP concentration were determined. As shown in Figure 2, mutation of the R to an L in the first triad increased the EC_{50} for GC-A from 74 to 528nM, a 7-fold change. In contrast, the single amino acid substitution in the second divergent triad (M-BNP) did not significantly change the Ec_{50} (74 nM vs. 62 nM) for GC-A activation. A peptide containing both substitutions (LM-BNP) reduced the activation similarly to the single L mutation alone and the difference between L-BNP and LM-BNP was not statistically significant (p value = 0.28).

Whole cell stimulation of human GC-B. Similar experiments were conducted on 293 cells stably expressing human GC-B to determine the ability of the substituted peptides to activate this receptor. As shown in Figure 2B, micromolar concentrations of wild-type BNP are required to activate human GC-B. The substitution of M for S in the second triad (M-BNP) had no effect on the concentration of BNP required to activate GC-B. However, mutation of R to L in the first triad (L-BNP) lowered its EC_{50} from 2,460 nM to 475 nM, a 5-fold decrease. Similar, reductions in the EC_{50} were observed for the LM-BNP double mutant. These results are inversely correlated with the reductions in EC_{50} for GC-A activity shown in the top portion of Figure 2.

Guanylyl cyclase-activating activity of peptides. To directly evaluate the effect of the amino acid substitutions on the ability of BNP to activate GC-A and GC-B, guanylyl cyclase assays were

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performed on crude membranes from 293 cells expressing either GC-A or GC-B. Adding membranes to mixtures containing 1 mM Mg^{2+}GTP and various concentrations of wild type or mutant natriuretic peptides initiated the reactions. Cyclic cGMP formed over a three-minute period was determined and plotted as pmol of cGMP/mg protein/min measured per individual concentration of BNP (Figure 3). Similar to the whole cell stimulation assays, L-BNP and LM-BNP displayed reduced potency for GC-A, whereas the EC_{50} for activation of GC-A by M-BNP was similar to wild type BNP. However, in this broken cell assay, the doubly substituted peptide (LM-BNP) was a less potent activator of GC-A than the singly substituted analog (L-BNP).

Wild type CNP was the best activator of human GC-B but both L-BNP and LM-BNP were better activators of GC-B than M-BNP and wild type BNP. The calculated EC_{50}s for L-BNP, LM-BNP, M-BNP and wild type BNP were 661 nM, 520 nM, 8,189 and 8, 481 nM, respectively. In contrast to the whole cell activation experiment, L-BNP was a better activator than LM-BNP.

Effect on GC-A binding. Competition ^{125}I-ANP whole cell binding experiments were performed to determine whether the changes in guanylyl cyclase activity were due to modulations in GC-A affinity or the ability of the bound ligand to induce conformational changes necessary for GC-A activation. We found that the binding profile mirrored the results from the whole cell cGMP elevation and guanylyl cyclase assays. M-BNP bound GC-A similarly to wild-type BNP, whereas L-BNP and LM-BNP bound approximately 5-fold less tightly (Fig 4). The IC_{50} increased from 5 nM for wild type BNP to 21 nM for L-BNP and 25 nM for LM-BNP. Both values were statistically different from wild type BNP. However, the IC_{50} obtained for M-BNP

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was not statistically different from the wild-type BNP. These data indicate that the changes in GG-A and GC-B activation result from changes in the affinity of the peptides for the receptors.

Effect on NPR-C binding. BNP is primarily removed from the circulation by NPR-C mediated internalization and degradation. The clearance receptor binds all three natriuretic peptides similarly. However, BNP has been shown to bind about 10-fold less tightly to NPR-C, which may contribute to the increased half-life of BNP compared to ANP and CNP (Suga et al., 1992). To directly measure the affinity of each peptide to NPR-C, the ability of the wild type or mutant forms of BNP to compete for ^{125}I-ANP binding to 293 cells stably expressing human NPR-C was investigated (Figure 5). In this assay, wild type BNP bound to NPR-C with a dissociation constant of 3.0 nM while the derivative peptides had dissociation constants of 2.0, 5.2, and 6.4 nM for L-BNP, M-BNP and LM-BNP, respectively. Unlike GC-A, the dissociation constants for wild type BNP and L-BNP were not significantly different at $p < 0.05$.

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DISCUSSION

In this report, we have identified a single Arg to Leu substitution in BNP that increases the EC_{50} for GC-A 7-fold while reducing the EC_{50} for GC-B by 5-fold. In contrast, a Met for Ser mutation in the second divergent triad had no effect on activation of either cyclase. A peptide containing both substitutions (LM-BNP) behaved like L-BNP in whole cell activation assay, although in broken cell assays L-BNP was a better activator of both cyclases, consistent with L-BNP being more stable under these conditions than LM-BNP. Competition binding experiments indicated that the increased EC_{50} for GC-A was due to reduced binding of L-BNP to the receptor. The analogous CNP binding experiments were not conducted due to lack of availability of a high quality ^{125}I-CNP tracer, but it is likely that the reduced EC_{50} for GC-B results from affinity changes as well. Interestingly, the affinity of wild type BNP and L-BNP to NPR-C was not significantly different, which indicates that the Leu substitution in BNP is a key binding determinant for the guanylyl cyclase but not clearance receptors.

These data are related to previous results from our group on the activation of GC-A and GC-B by CD-NP (Dickey et al., 2008). CD-NP is designer natriuretic peptide consisting of the 15-residue carboxyl-terminus of dendroaspis natriuretic peptide appended to carboxyl-terminus of CNP (Lisy et al., 2008) (Fig. 1). In a recent clinical trial, CD-NP demonstrated natriuretic and aldosterone-suppressing properties without inducing excessive hypotension (Lee et al., 2009). We found that CD-NP bound GC-A tighter than CNP, which indicates that the carboxyl-terminal extension increases the affinity of CNP to human GC-A. Additional studies investigated the effect of substituting all three amino acids from the first variable region of BNP into CD-NP

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(Fig. 1). We found that substitution of RKL from BNP for LKL in CNP converted CD-NP into a potent GC-A agonist. In fact, this peptide was more potent and bound GC-A more tightly than wild type BNP. In contrast, the substitution of SSS from BNP for GSM in CNP in the second variable triad only slightly increased the affinity and potency of CD-NP for GC-A. As for L-BNP, reciprocal reductions in the potency of the mutant CD-NP peptides for GC-B were also observed. However, although the mutation in the second triad only slightly decreased potency for GC-A, it dramatically decreased potency for GC-B.

The data presented here are also consistent with a previous report by Furuya and colleagues who found the replacing the LKL region with the analogous triad from ANP (GRM), markedly reduced CNP-dependent cGMP elevations in rat smooth muscle cells (Furuya et al., 1992). They also found that individual substitutions in the second triad had little effect on rat GC-B activation, which is consistent with our results showing that the EC_{50} for activation of human GC-B by BNP and M-BNP are similar.

In summary, we have identified a single mutation in BNP that differentially regulates binding and activation of GC-A and GC-B without affecting binding to NPR-C. Future studies will focus on the effect of this mutation on the half-life of the peptide.

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FOOTNOTES

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LEGENDS FOR FIGURES

FIGURE 1. **Structure of wild type and derivative natriuretic peptides**. The sequence of human ANP, BNP, CNP and derivative peptides are shown with identical amino acids shaded and mutated amino acids bolded and underlined.

FIGURE 2. **Mutation of Arg17 to Leu in BNP increases and decreases the EC_{50} for GC-A and GC-B, respectively.** Confluent 293neo cells stably expressing either human GC-A or human GC-B were incubated with increasing concentrations of ligand for 3 min and then cellular cGMP were determined. *Top*, human GC-A (p values: wt vs. L-BNP = 0.029; wt vs. M-BNP = 0.69; wt vs. LM-BNP = 0.079; L-BNP vs. LM-BNP = 0.28). *Bottom*, human GC-B (p values: wt vs. L-BNP = 0.085; wt vs. M-BNP = 0.19; wt vs. LM-BNP = 0.07; L-BNP vs. LM-BNP = 0.0008).

FIGURE 3. **Mutation of Arg17 to Leu in BNP increases and decreases the EC_{50} for GC-A and GC-B, guanylyl cyclase activity, respectively.** Crude membranes from 293neo cells stably expressing human GC-A or human GC-B were incubated for 3 min. at 37°C in the presence or absence of increasing concentrations of various BNP-derived peptides. Guanylyl cyclase activities were determined and plotted as a function of BNP concentrations. *Top*, human GC-A. *Bottom*, human GC-B.

FIGURE 4. **Mutation of Arg17 to Leu in BNP decreases affinity for human GC-A.** 293neo cells stably expressing human GC-A were incubated for 1 hr at 4°C with 50 pM ^{125}I-ANP in the

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presence or absence of increasing concentrations of unlabeled ligand. After washing to remove nonspecific binding, the amount of bound radioligand was quantitated in a gamma counter. The data points represent the mean +/- the standard error of the mean of three separate experiments assayed in triplicate. (p values: wt vs. L-BNP = 0.028; wt vs. M-BNP = 0.26; wt vs. LM-BNP = 0.042; L-BNP vs. LM-BNP = 0.45)

FIGURE 5. **Wild type BNP and L-BNP bind NPR-C with similar affinities.** 293neo cells stably expressing human NPR-C were incubated ^{125}I-ANP in the presence or absence of increasing concentrations of unlabeled ligand as described above. The amount of specific binding is shown. The data points represent the mean +/- the SEM of two separate experiments assayed in triplicate. (p values: wt vs. L-BNP = 0.058; wt vs. M-BNP = 0.016; wt vs. LM-BNP = 0.002; L-BNP vs. LM-BNP <0.0001)

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FIGURE 1

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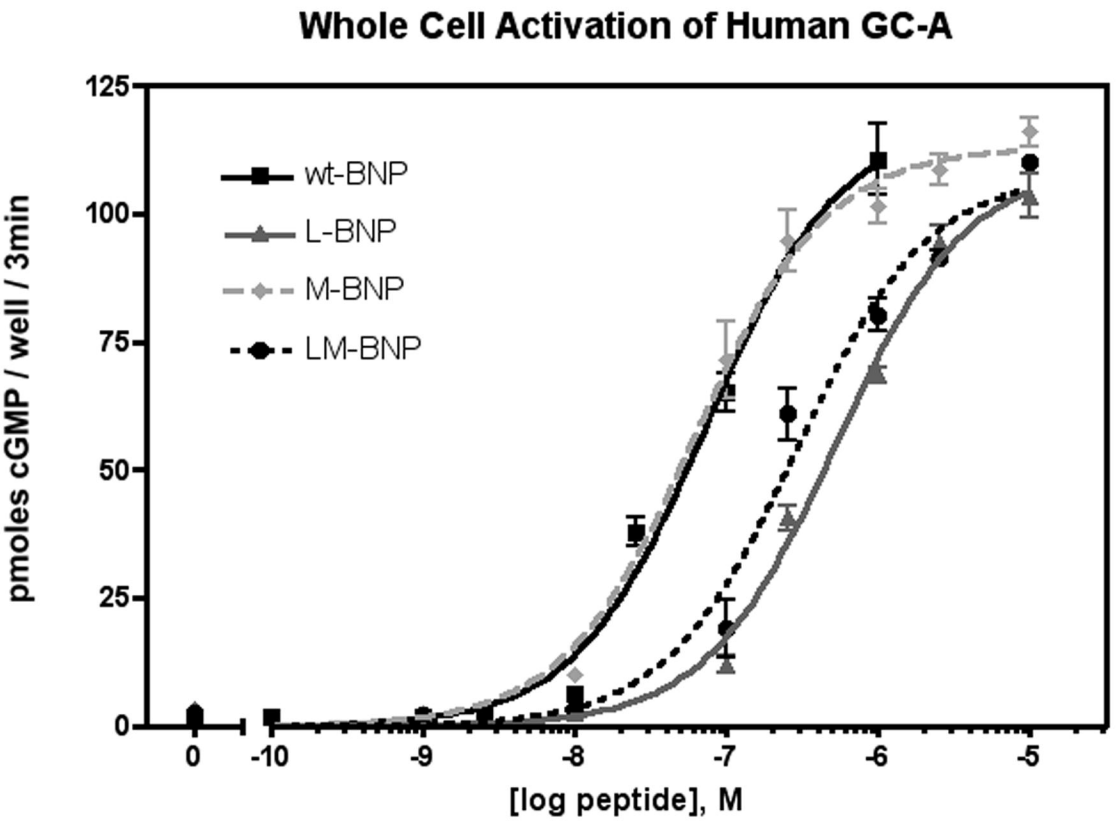
Figure 2.



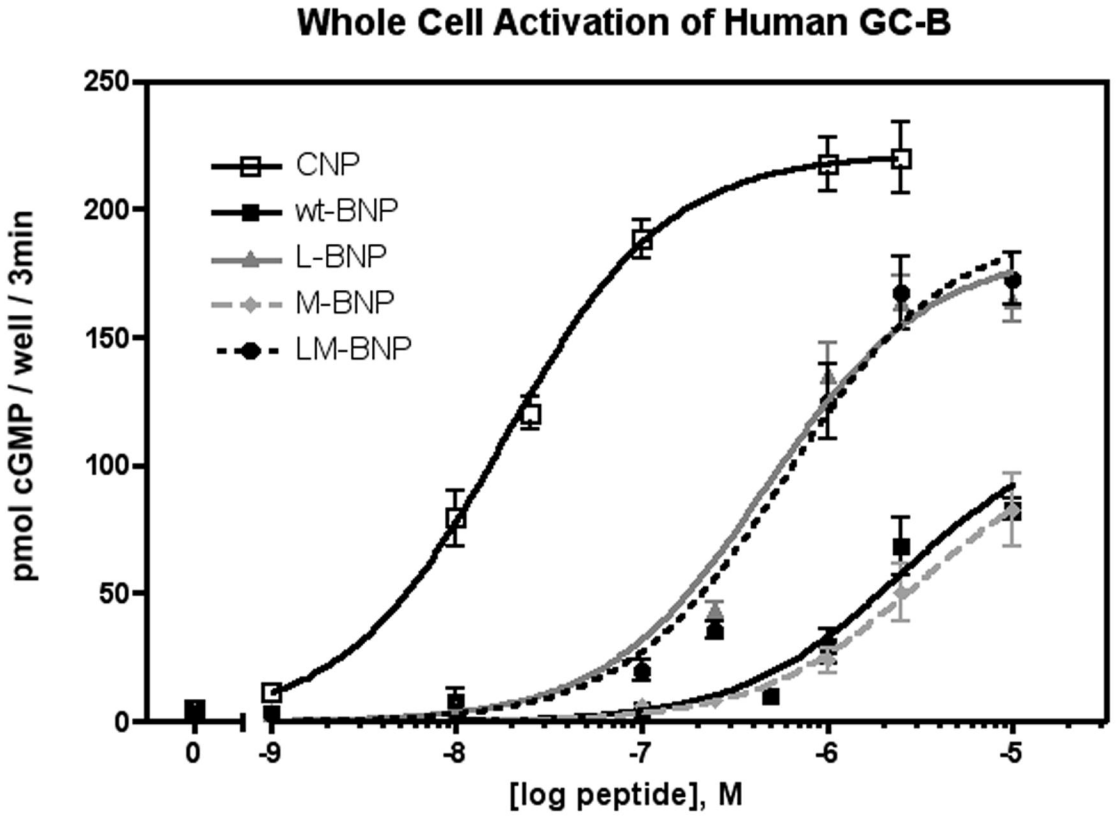


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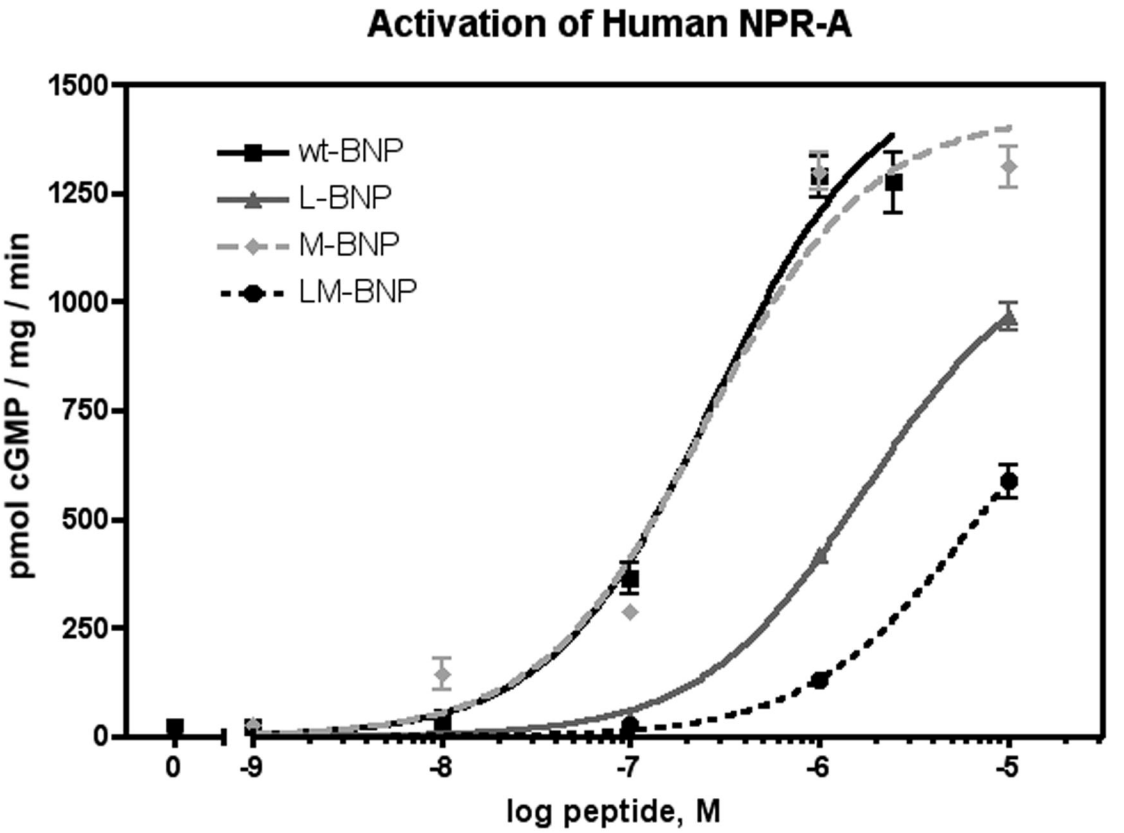
Figure 3.



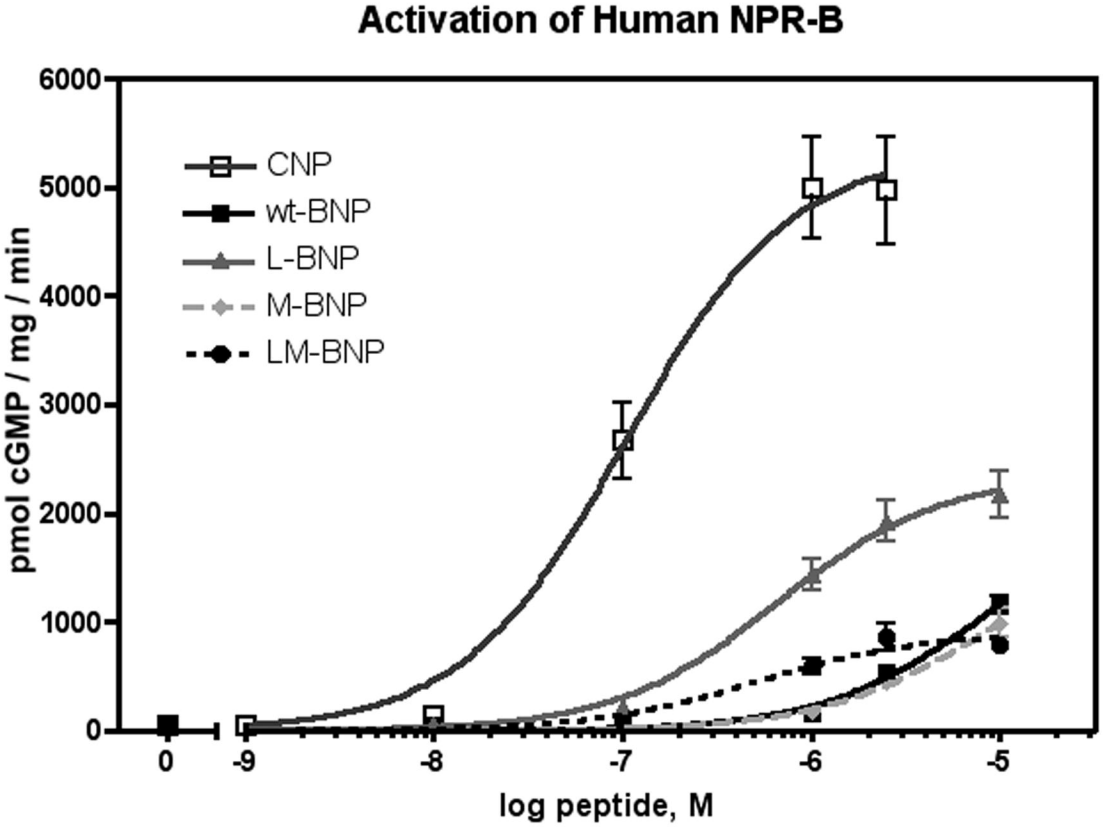


Figure 4

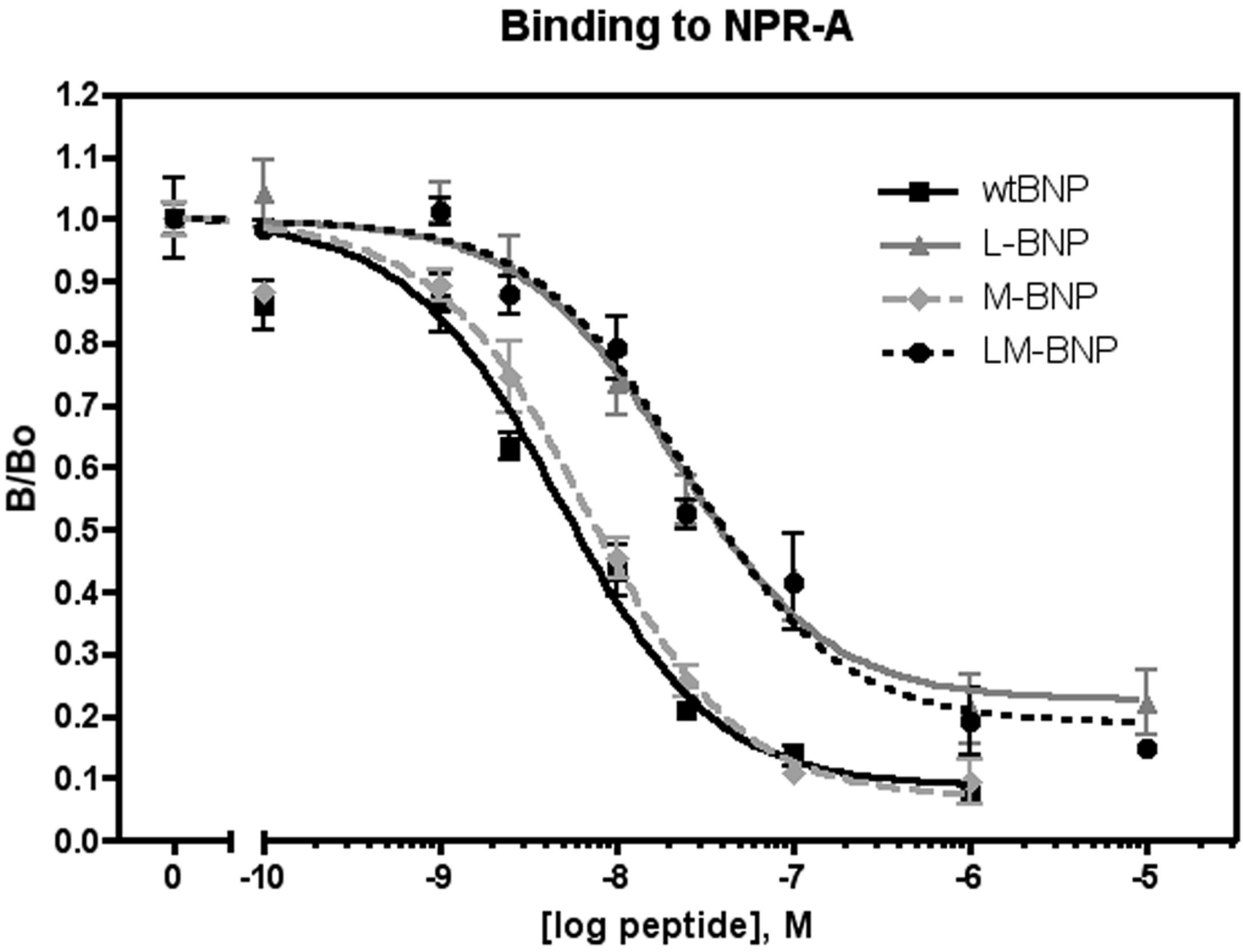

Binding to NPR-A
B/Bo
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
-10
-9
-8
-7
-6
-5
[log peptide], M
wtBNP
L-BNP
M-BNP
LM-BNP

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Figure 5