Mutations in B-type natriuretic peptide mediating receptor-A selectivity

Jill R. Schoenfeld[a], Jeff Y.K. Tom[b], David G. Lowe[a],*

[a]Department of Cardiovascular Research, Genentech, Inc., 460 Point San Bruno Blvd., South San Francisco, CA 94080, USA
[b]Department of Bioorganic Chemistry, Genentech, Inc., 460 Point San Bruno Blvd., South San Francisco, CA 94080, USA

Abstract Libraries of monovalent display-phage expressing mutant human B-type natriuretic peptide (hBNP) were used to identify variants that preferentially bind natriuretic peptide receptor-A (NPR-A) compared to receptor-C (NPR-C). Position 19 was a significant determinant of receptor specificity for hBNP display phage. The synthetic hBNP variant S19R had a 265-fold improved NPR-A binding over NPR-C, analogous to the atrial natriuretic peptide (ANP) specificity mutation G16R. Mutation of the last three residues of the hBNP disulfide ring, G23F/ L24W/G25R, resulted in about 9-fold improved selectivity. The analogous mutations in ANP decreased NPR-A binding, suggesting divergence in the mechanism of NPR-A recognition. © 1997 Federation of European Biochemical Societies.

Key words: B-type natriuretic peptide; Atrial natriuretic peptide; Natriuretic peptide receptor; Receptor/hormone interaction; Phage display; Mutagenesis

1. Introduction

B-type natriuretic peptide (BNP) is a member of a family of related polypeptide hormones that includes ANP and CNP [1,2]. Both ANP and BNP are produced by atrial myocytes, and by ventricular myocytes under conditions of increased load [3,4]. Two receptors have been described for these cardiac hormones. NPR-A, or guanylyl cyclase-A, directly synthesizes the second messenger cGMP in response to ligand binding [5]. The second receptor, NPR-C or the clearance receptor, functions in the internalization and degradation of bound ligand, and may be involved in signal transduction [6,7].

In this report we investigated the determinants of hBNP receptor selectivity by monovalent phage display [8,9] of libraries of mutant hBNP molecules. Using receptor extracellular domain-IgG fusions as binding targets we selected for hBNP variants that selectively bound A-IgG in the presence of excess competing C-IgG. Our results indicate similarities and differences between receptor-selectivity mutations in hANP and hBNP, and suggest that NPR-A recognition of these homologous polypeptides is not identical.

*Corresponding author. Fax: (1) (415) 225-6327.
E-mail: lowe@gene.com

Abbreviations: hANP, human atrial natriuretic peptide; ANP(FWR22), hANP(G20F, L21W, G22R); hBNP, human B-type natriuretic peptide; BNP(GR14), hBNP(R13G, K14R); BNP(R19), hBNP(S19R); BNP(FWR25), hBNP(G23F, L24W, G25R); NPR, natriuretic peptide receptor; A-IgG, NPR-A extracellular/IgG heavy chain fusion protein; C-IgG, NPR-C/IgG fusion protein

2. Materials and methods

2.1. Construction of BNP display phage

Phagemid encoding the hBNP fusion with M13 gIII was made from the ANP phagemid vector [9] by site-directed mutagenesis [10]. Libraries of mutant hBNP molecules with randomization of from 3 to 4 codons were constructed using primer-directed mutagenesis with the following oligonucleotides: positions 11–14, 5′-GTGCA-GGGTTCTGGTTGCNNSNNSNNSNNSATGGATCGTATCAGC-TCC-3′; positions 15–18, 5′-GGTTGCTTCGGCAGAAAGNNSN-NSNN SNNSAGCTCCAGCAGCGGTCTC-3′; positions 19–22, 5′-AAGATGGATCGTATCNNSNNSNNSNNSGGTCTCGGGTG-CAAAGTG-3′; positions 23–25, 5′-CGTATCAGCTCCAGCAG-CNNSNNSNNSTGCAAAGTGCT GCGTCGT-3′. The libraries were made with template DNA that contained frameshift mutations to prevent hBNP background in the initial library.

2.2. Competitive panning for hNPR-A selective hBNP variants

Competitive selection of hBNP variants with hNPR-A and hNPR-C was done with receptor extracellular domain-IgG fusions, referred to as A-IgG and C-IgG [11]. Phage libraries went through one round of enrichment for A-IgG binding before introducing negative competitive binding pressure with C-IgG. For both enrichment and competitive selection, wells of Nunc Maxisorb microtiter plates (catalog no. 4-39454) were coated with 100 μl of 2 μg/ml rabbit anti-human Fc antibody (Jackson ImmunoResearch catalog no. 309-006-008) in coat buffer (50 mM carbonate, pH 9.6) overnight at 4°C. The coat solution was discarded and the wells were incubated for 1 h at room temperature with 25 mM carbonate pH 9.6 in 5% skim milk to block non-specific binding. After washing with 0.01% (v/v) Tween 20 in phosphate buffered saline (PBS), A-IgG was added at 1 μg/ml in binding buffer [2% (w/v) skim milk in phosphate buffered saline (PBS)] for 1 h at room temperature then washed once in binding buffer. For enrichment 10 μl of phage library stock (250 fold concentrated from growth medium, stored in PBS at 4°C) was diluted to 100 μl in binding buffer and incubated in the A-IgG coated wells for 2 h at room temperature. After washing 20–30 times the bound phage was recovered by adding 100 μl of 0.2 M glycine pH 2.0 to the wells for 1 m and neutralizing with 100 μl of 1.0 M Tris base. The recovered phage was mixed with 1.0 ml of *E. coli* XL1-blue cells (Stratagene) in log growth phase, and grown for 80 min at 37°C with shaking. KO7 helper phage was added and the culture was grown for an additional 30 min. The infected culture was transferred to 25ml of 2YT media (with 50 μg/ml carbenicillin and 5 μg/ml tetracycline) and grown overnight at 37°C. Phage particles were recovered by two polyethyleneglycol/NaCl precipitations and resuspended in 100 μl of PBS for the next round of selection. Competitive selection was done in seven rounds, with increasing amounts (20 nM to 200 nM) of C-IgG included at successive cycles. Individual clones were picked and the measurement of phagemid receptor affinity by ELISA was essentially as described [9] with the following modifications: phage binding buffer was 2% (w/v) skim milk in PBS and the anti-phage antibody was Sheep anti-M13 horse radish peroxidase conjugate (Pharmacia).

2.3. Peptide affinities and agonist potency

Membranes from 293 cell lines stably expressing NPR-A [12] or NPR-C [9] were prepared and tested for ligand binding essentially as described [13]. Briefly, membrane samples were diluted in 50 mM HEPES, 5 mM MgCl$_2$, 100 mM NaCl, 0.1 mM EDTA, 0.2% (w/v) BSA, 1 μM phosphoramidon, and added to an equal volume of synthetic peptide plus 30 pM [^{125}I]rANP (3000 Ci/mmole, Amersham

Corp). The binding was allowed to go for 2 h at room temperature in 96 well U-bottom polypropylene microtiter plates (Sigma), with gentle agitation. Bound ligand was separated from free prior to scintillation counting, as previously described [13]. Assays were set up in duplicate and repeated three times. Concentration-response experiments for stimulation of cGMP production in 293 cells expressing hNPR-A were set up and analyzed as before [14]. Stimulations were performed in triplicate and assayed in duplicate for cGMP production. Results from competition binding and cGMP stimulation experiments were analyzed by a four parameter logistics equation.

3. Results

3.1. Identification of NPR-A selective phagemid clones

Libraries of mutant hBNP display phage were constructed by primer directed mutagenesis and subjected to seven rounds of competitive panning to identify NPR-A selective variants with reduced NPR-C binding. The 32-amino acid hBNP sequence (Fig. 1) was divided into mutagenesis windows covering the N- and C-terminal tail sequences and the 17 amino acid ring. No receptor selectivity mutations were found in the tail sequences (data not shown), however 3 of the 4 mutagenesis windows in the disulfide ring did provide amino acid changes imparting receptor selectivity (Table 1). Individual phagemid clones were sequenced and tested for binding to A-IgG (Table 1). Representative clones that retained A-IgG binding were then tested for preferential receptor binding by phage ELISA (Fig. 2). Mutagenesis window A was sorted to two sequences, only one of which, sequence 1, showed binding to A-IgG by phage ELISA (Table 1, Fig. 2). This clone had the misincorporation mutation S19R outside of the mutagenesis window, directly analogous to the G16R misincorporation mutation found in ANP display phage [9]. Interestingly this mutation was accompanied by mutations R13G and K14R that change this hBNP sequence to the corresponding ANP sequence (Fig. 1). By phage ELISA this sequence had reduced C-IgG binding (Fig. 2).

Mutagenesis window B did not provide NPR-A selective clones, whereas window C was also characterized by the presence of a misincorporation mutation, at position 14, outside the target mutagenesis region (Table 1). Window C clones had a basic amino acid substituted at either or both of position 19 or 20, and a misincorporation change in the basic amino acid K14 to a polar residue. Phagemid clones from this mutagenesis window retained A-IgG binding and had reduced binding to C-IgG (Table 1, Fig. 2).

The last 3 amino acids in the ring, covered in mutagenesis window D, were selected to an apparent consensus sequence unlike anything previously seen in the natriuretic peptides (Table 1). The clones obtained were dominated by the mutations MWR and FWR. These phagemid clones all retained A-IgG binding and the most abundant sequences had significantly reduced binding to C-IgG (Table 2, Fig. 2).

3.2. Receptor affinities of synthetic hBNP variants

Synthetic peptides incorporating mutations identified by

hANP   ¹S L R R S S C F G G R M D R I G A Q S G L G C N S F R Y²⁸
hBNP   ¹S P K M V Q G S G C F G R K M D R I S S S S G L G C K V L R R H³²

Fig. 1. Comparison of human ANP and BNP sequences. Identical residues are boxed. The 17 amino acid ring-forming disulfide bond is indicated by a line at the top.
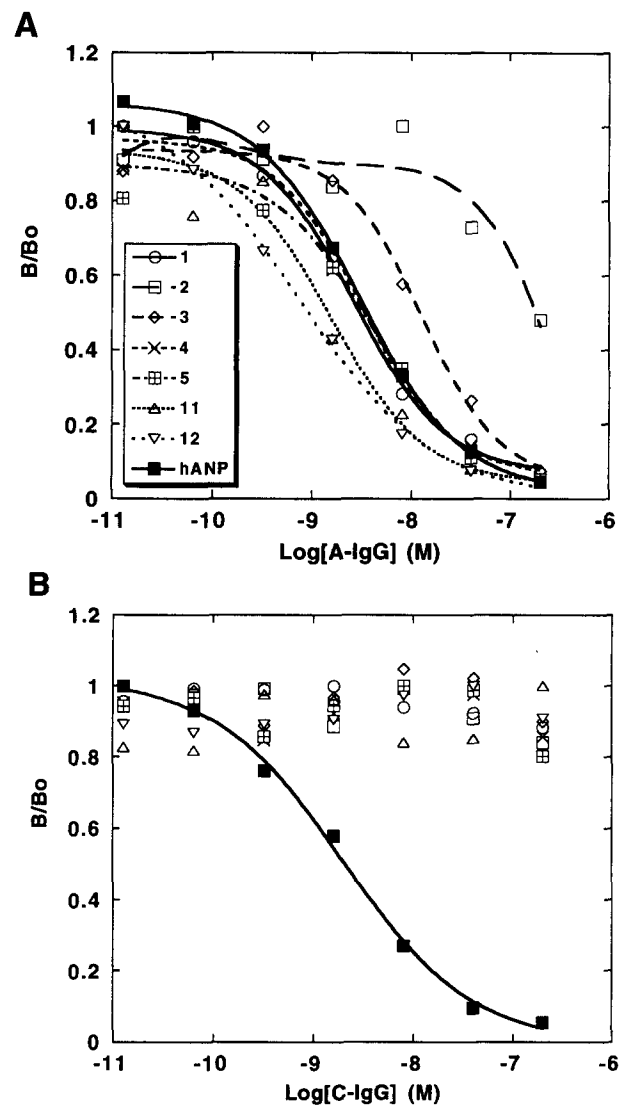

A

B

Fig. 2. Phage ELISA. Individual phagemid clones were incubated with immobilized A-IgG in the presence of increasing concentrations of solution-phase A-IgG (A) or C-IgG (B). The key in panel A corresponds to individual phagemid sequences in Table 1.

monovalent phage display were prepared in order to confirm the results of phage ELISA assays on receptor selectivity. Using hBNP and hANP as reference peptides, we compared the effect of the mutations in competitive binding assays using membranes from 293 cells expressing recombinant hNPR-A or hNPR-C (Table 2). The first series of peptides, BNP(GR14/R19), BNP(GR14), and BNP(R19), were designed to address the role of mutations in window A combined with the Arg19 misincorporation mutation at position 19. The position 19 substitution also addresses the specific role of Arg substitution at this position in window C. The combined mutation in BNP(GR14/R19) showed decreased NPR-C binding, about 15 fold. In contrast BNP(GR14), changing hBNP to the corresponding hANP sequence, showed decreased affinity to both receptors. Receptor selectivity from window A is then coming from the S19R misincorporation mutation; BNP(R19) has both increased affinity to NPR-A and decreased NPR-C binding, resulting in an overall relative improvement in NPR-A preference of 265 (Table 2).

The mutation FWR was picked as representative of the

Table 1
Sequences of selected hBNP variants[a]

Clone Number	hBNP Randomization Window				Relative[b] A-IgG Binding (mutant/ hANP)	# of clones
	A 10	B 15	C 20	D 25		
	©(F)(G)(R)(K)(M)(D)(R)(I)(S)(S)(S)(G)(L)(G)(C)					
1	G R		R		**1.6**	10
2	G P N R				**>20**	4
3		T	R S Q		**4.2**	10
4		N	R H L		**1.0**	1
5		N	A R E		**1.3**	1
6		N	N K T		4.2	3
7		N	G R A		4.8	1
8		Q	R H S T		4.0	2
9		N	R		3.5	1
10		T	R H G H		5.0	2
11				M W R	**1.0**	7
12				F W R	**0.8**	7
13				M Y R	2.4	2
14				L W R	2.0	3
15				M F R	3.0	1

Four libraries of mutant BNP display phage were created by randomization of three or four adjacent codons in the disulfide bonded ring sequence of BNP (randomization windows A–D). Display phages were enriched by one round of panning on the NPR-A ECD fusion protein A-IgG, then subjected to seven rounds of competitive panning with immobilized A-IgG and solution phase C-IgG. 14–20 individual clones from each selection were sequenced and analyzed for receptor binding by phage ELISA (see corresponding clone numbers in Fig. 2). Selectants from randomization window B all had reduced binding to A-IgG.
[a]The 17 amino acid sequence of the disulfide linked hBNP ring is shown in single letter code. For the complete hBNP sequence see Fig. 1.
[b]Values in bold are derived from the data in Fig. 2A.

selectivity changes found in window D (Table 1). The synthetic peptide BNP(FWR25) showed both enhanced NPR-A binding and decreased NPR-C binding for an overall 8.7-fold increase in NPR-A preference. Whereas the native 23, 24, 25 sequence GLG is conserved in hBNP and hANP (Fig. 1), the FWR mutation does not have analogous effects on ANP. The peptide ANP(FWR22) has decreased binding to NPR-A (11.5-fold) and a minor effect on NPR-C binding (Table 2), demonstrating that these changes cannot be engrafted to hANP.

To determine if further improvement in receptor selectivity can be achieved, the combined mutant molecule BNP(R19/FWR25) was synthesized. Compared to each mutation alone, R19 or FWR25, the combined mutations were intermediate in improved NPR-A affinity. There is further impairment of NPR-C binding with the combined mutations, but the net effect on receptor selectivity (NPR-C/NPR-A preference) was comparable to the single S19R substitution in BNP(R19) (Table 2).

3.3. Agonist potency of BNP variants
Native ligands and synthetic variants were tested for agonist potency in stimulating NPR-A signal transduction. Concentration-response or single-dose stimulations of cGMP production were done with cultured 293 cells expressing recombinant hNPR-A (Fig. 3, Table 3). Consistent with the binding data, BNP(GR14) had impaired activity, whereas BNP(GR14/R19) had restored activity (Fig. 3B). Interestingly, BNP(R19) had enhanced stimulation of cGMP production which was apparently lost in the combination mutant BNP(GR14/R19). Enhanced stimulation was also observed for the other variants tested (Fig. 3A), with from 15 to 29% higher plateau cGMP levels in the concentration response assay (Table 3).

Enhancements in NPR-A affinity (Table 2) did not necessarily correlate with ED_{50} for agonist potency (Table 3). Both BNP(R19) and BNP(FWR25) had insignificant improvements in agonist potency compared to hBNP, whereas BNP(R19/FWR25) had improved affinity but approximately 2-fold lower agonist potency (Table 3). For ANP(FWR22) we observed both enhanced maximal stimulation of cGMP and about 15-fold reduced agonist potency (Table 3) compared to a 6-fold reduction in binding.

4. Discussion

In this report we describe the results of a competitive panning experiment in which immobilized A-IgG and solution phase C-IgG were used to identify NPR-A selective hBNP variants expressed in libraries of mutant display phage. Several classes of mutations may be identified by this strategy including receptor selectivity, increased affinity for NPR-A, or increased secretion expression in E. coli[9]. Previous work

Table 2
Receptor binding data for natriuretic peptide mutants

Peptide	Receptor binding IC50 (pM)		Relative affinity[a] (IC50 Mutant/IC50 WT)		Fold-change in selectivity[b]
	NPR-A	NPR-C	NPR-A	NPR-C	
hANP	44 ± 13	198 ± 90	1	1	1
hBNP	645 ± 298	1791 ± 145	1	1	1
BNP(GR14/R19)	518 ± 72	$(2.8 ± 1) \times 10^{3}$	0.8	15.6	19.5
BNP(GR14)	$(4.4 ± 0.6) \times 10^{4}$	$(1.4 ± 1.3) \times 10^{4}$	68	7.8	0.11
BNP(R19)	155 ± 55	$(9.5 ± 1.5) \times 10^{4}$	0.2	53	265
BNP(FWR25)	378 ± 136	9363 ± 2067	0.6	5.2	8.7
ABP(FWR22)	507 ± 118	295 ± 57	11.5	1.5	0.13
BNP(R19/FWR25)	287 ± 27	$(1.7 ± 1.2) \times 10^{4}$	0.4	95	237

Competitive binding experiments were done with membranes from human embryonic kidney 293 cells expressing full length hNPR-A or NPR-C, with serial dilutions of peptides and [^{125}I]ANP as tracer. A single class of binding site was observed in each case. Data are the average of triplicate measurements ± standard deviation.
[a]Relative affinity is based on hANP as a reference for ANP(FWR22), and hBNP for each of the BNP peptides.
[b]Fold-change in selectivity is the ratio of relative affinities (NPR-C/NPR-A), with a number > 1 indicating greater preference for NPR-A relative to the native peptide.

with libraries of mutant ANP display phage found that receptor selectivity mutations were confined to the 17 amino acid disulfide bonded ring [9]. We obtained similar results with hBNP using the same strategy for mutagenesis window selection. The choice of alternative mutagenesis windows may however give a different result.

In two mutagenesis windows we found misincorporation mutations (positions 14 and 19) that occurred as a result of low frequency errors in the mutagenesis oligonucleotide. This is in contrast to the misincorporation mutation found in ANP as a result of polymerase error [9], and highlights the sensitivity of the selection in identifying mutations that confer selectivity.

Our results demonstrate that a common feature shared by hBNP and hANP is the receptor selectivity afforded by Arg substitution at positions 19 or 16, respectively [9]. In both

Table 3
Peptide agonist potency on hNPR-A

Peptide	ED$_{50}$ (nM)	Maximum cGMP[a] (pmoles/well)
hANP	0.43 ± 0.04	1564 ± 21
hBNP	2.73 ± 1.12	1476 ± 88
BNP(GR14/R19)[b]	1–10	N.D.
BNP(GR14)[b]	> 100	N.D.
BNP(R19)	1.67 ± 0.24	1886 ± 44
BNP(FWR25)	1.77 ± 0.03	1842 ± 6
ANP(FWR22)	2.00 ± 0.45	1794 ± 72
BNP(R19/FWR25)	6.8 ± 1.1	2014 ± 59

Monolayer cultures of 293 cells stably expressing hNPR-A were stimulated with peptides for 10 min and 37°C, and cGMP produced was measured by radioimmunoassay. ED$_{50}$ and Maximum cGMP values are derived from the concentration-responses and single concentration stimulations plotted in Fig. 3.
[a]N.D., not determined.
[b]Based on single dose estimations from Fig. 3B.

cases this substitution was detected by selection of a misincorporation mutation outside the target mutagenesis window in addition to selection from within a window. In the case of hBNP this misincorporation mutation occurred in conjunction with two substitutions within the mutagenesis window that change the hBNP sequence to that of hANP. Our results with synthetic peptides show that these changes in window A are not involved in receptor selectivity and may be required for secretion expression of the Arg 19 hBNP fusion protein in E. coli. Window C mutations (Table 1) resulted in the selection of basic residues at position 19 and/or 20, with a corresponding misincorporation mutation at position 14, Lys substituted for a polar residue. As with ANP, the basic nature of the polypeptide (pI = 12) may not be favorable for optimal secretion expression, with the result that some selected mutations may in fact balance charge effects of selectivity changes.

Although there are clearly similarities between hBNP and hANP in the Arg 19 or 16 mutation, the selectivity changes identified in the last mutagenesis window have no analog in ANP. These last 3 amino acids of the disulfide ring are conserved in hBNP and hANP (Fig. 1), as well as C-type natriuretic peptide [2]. We can conclude from this result that there are different determinants of receptor specificity for hBNP and hANP in binding and activation of NPR-A. Whereas mutagenesis of these residues in hANP did not identify receptor selectivity mutations, the converse is true at the beginning of the ring. In this region, covering the first 4 residues of the ring, receptor selectivity mutations were identified for ANP [9], but our experiment (Table 1, window A) did not identify any hBNP selectivity mutations in this region. These data highlight fundamental differences in the mode of ligand recognition by NPR-C and NPR-A. This conclusion is also true for cross-species comparisons of NPR-A and NPR-C, with divergent modes of recognition for both ANP and BNP variants [14–16]. Together these observations suggest that each receptor/ligand pair will have some unique structural determinants of specificity and affinity, perhaps reflected in a variable contour and different side-chain contacts at the receptor/ligand interface.
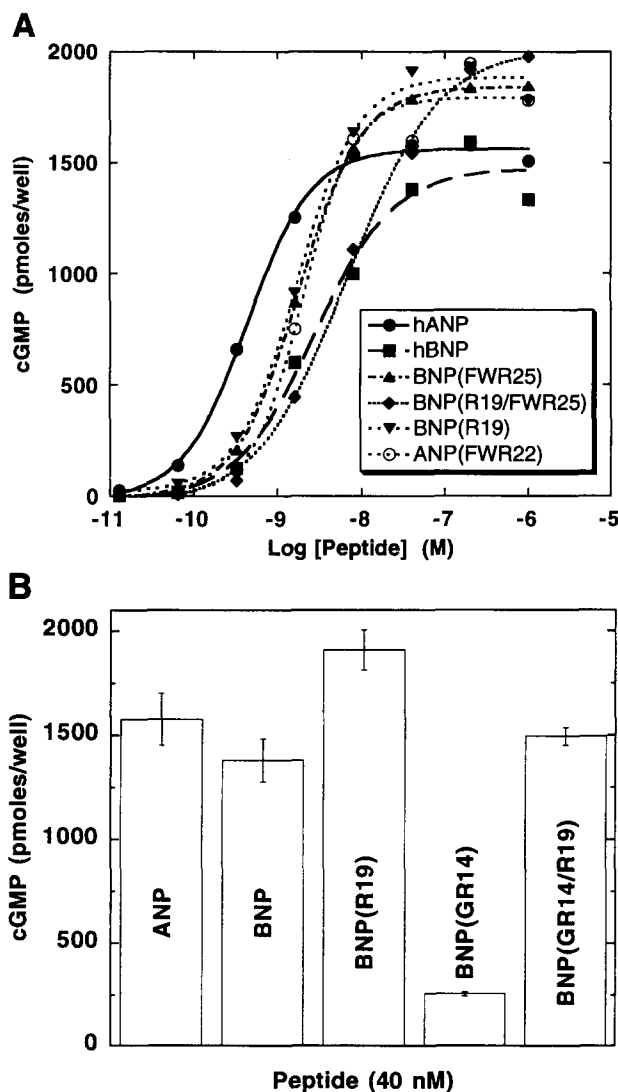

Fig. 3. Stimulation of cGMP production. Concentration-response measurements for cGMP production (A) were performed with ANP, BNP, and analogs (see legend at right). Results are the average of 3 determinations, and are plotted as a four parameter curve fit (see Section 2). Single-dose stimulation experiments (B) were done with several analogs at 40 nM concentration. Values plotted for ANP, BNP, BNP(FWR), BNP(S19R, FWR), BNP(S19R) and ANP(FWR) are taken from panel A.

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