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Vasonatrin peptide stimulates both of the natriuretic peptide receptors, NPRA and NPRB

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ABSTRACT

Vasonatrin peptide (VNP) is an active cardiovascular factor and a novel synthetic natriuretic peptide with unknown natriuretic peptide receptor (NPR) binding properties. We set out to design binding models of NPRA/VNP and NPRB/VNP, and then assessed their recognition and binding affinities using molecular dynamics. Molecular dynamics analysis indicated decreases in the values of Van der Waals, electrostatic energy and potential energy of NPRB/VNP compared to NPRA/VNP. There was a 25% increase in H-bond formation between VNP and NPRB. The cGMP stimulated by VNP in NPRB-transfected HEK-293 cells was 11-fold higher than that of NPRA. We therefore demonstrated that VNP binds with both NPRA and NPRB, but with a preference for NPRB.

1. Introduction

Natriuretic peptides (NPs) are important hormones in cardio-renal homeostasis [1]. There are three natural human NPs: atrial natriuretic peptide (ANP, 28 amino-acids) [2], brain natriuretic peptide (BNP, 32 amino acids) [3] and C-type natriuretic peptide (CNP, 22 amino acids) [4]. The different NPs show structural similarities but have genetically distinct peptide-binding properties. All NPs share a conserved 17-residue ring structure, formed by an intra-chain disulfide bond between two cysteine residues, which is essential for their bioactivity [5]. Disruption of this ring structure by reductive cleavage of the conserved residues leads to loss of bioactivity [6]. NPs may either have one (CNP) or two different tails in the NH_2 and COOH terminus, which causes differences in receptor binding affinity [5].

Vasonatrin peptide (VNP), a novel synthetic member of the NP family that is a chimera of CNP and ANP, is characterized by the 22-amino-acid ringed structure of CNP along with the COOH terminus of ANP [7]. VNP is reported to have greater potential to relax the abdominal aorta and celiac vein [8] and a stronger inhibitory effect on the proliferation of pulmonary artery vascular smooth muscle cells than ANP or CNP [9]. Both *in vitro* and *in vivo* studies suggested that VNP has beneficial effects on hypoxia-induced pulmonary hypertension [10–12] and there are no reports on the action of other NPs against this problem.

Most functions of NPs are mediated through elevated intracellular cyclic 3′,5′-guanosine monophosphate (cGMP) production upon binding to the natriuretic peptide receptors (NPRs), NPRA and NPRB [13]. ANP and BNP are NPRA ligands [14], while CNP exert its bioactivity *via* NPRB binding [15]. As a synthetic natriuretic peptide, VNP has cardiovascular bioactivity similar to the natural natriuretic peptides, ANP and CNP. So far, only NPRA has been reported to be the functional receptor subtype of VNP [16]. Based on its similarity with AC-NP [17] and CD-NP [18], VNP is predicted to be capable of binding to NPRB. In this study, we designed VNP binding models with NPRA and NPRB *in silico*, and carried out molecular dynamics on both models to demonstrate their recognition mechanism and binding affinity. The VNP stimulated cGMP production was also assessed in HEK-293 cells transfected with the NPRA and NPRB gene.

2. Materials and methods

2.1. Homology modeling of NPRA/VNP and NPRB/VNP

The complex structure of NPRA/ANP (PDB: 1T34) was downloaded from the Protein Database Bank and the amino acid-sequences of VNP (gi: 1829904) and NPRB (gi: 4580422) were downloaded from NCBI and saved in FASTA format.

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We designed the homology model of NPRA/VNP in accord with the template model of NPRA/ANP. Since there is no reported 3D structure for NPRB, we designed and verified the homology structure of NPRB with NPRA as the template structure, using Modeler 9v7 of the Discovery Studio 3.5 (Accelrys Inc., San Diego, CA). Then we obtained the complex model of NPRB/VNP using the same method as with NPRA/VNP.

2.2. Molecular dynamics (MD) simulations

The NPRA/VNP and NPRB/VNP complexes were proof-checked for any structural disorders, missing backbone atoms or side-chains, and incorrect connectivity or bond orders. All verified structures were used as MD simulation starting points. A full VNP–NPRs atom system was constructed as described in the Discovery Studio 3.5 solvation protocol with default parameters. All ionizable residues were assigned to their respective charge status corresponding to pH 7.5. The complex was then solvated in a water box using the Explicit Periodic Boundary solvation model with a minimum distance of 7.0 Å from the boundary to obtain an orthorhombic cell.

The system was subjected to the CHARMm force-field and relaxed by energy minimization (5000 steps of steepest descent and 5000 steps of conjugated gradient) with a protein harmonic restraint. The system was slowly driven from an initial temperature of 50 K to a target temperature of 300 K for 100 ps and equilibration simulations were run for 100 ps. MD simulations were performed at 100 ns using the NPT system at a constant temperature of 300 K. All the other parameters were set as defaults.

2.3. MD trajectory analysis

After 100 ns of MD, trajectory assessment was conducted every 10 ps, following the Discovery Studio 3.5 trajectory analysis protocol, the MD trajectory was determined for the structural properties, root mean-square deviation (RMSD), root mean-square fluctuation (RMSF) and potential energy. The interaction energy between VNP and NPRs was calculated using the interaction energy calculation protocol with all parameters set as defaults. The number of H-bond formed between VNP and NPRs was also quantified.

2.4. Peptide synthesis and bioactivity evaluation

VNP peptide was synthesized and purified by GLS Biochem Co., Ltd. (Shanghai, China). Confluent HEK-293 cells stably expressing either human NPRA or NPRB were used to evaluate the bioactivity of synthesized VNP (with a concentration gradient of 10^{-12}–10^{-5}mol/L) alongside the HEK-293 wild-type cells with no NPRA or NPRB as negative controls.

All cells were prepared and assayed as previously described [19]. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 g/mL streptomycin, and 200 g/mL G418. Cells were plated in 24-well plates. Prior to harvest, the cells were maintained in a serum-free medium for 4 h. The cells were incubated for 10 min at 37 °C in Dulbecco's modified Eagle's medium containing 25 mM HEPES (pH 7.4) and 1 mM 1-methyl-3-isobutylx-anthine. This medium was then replaced with the same medium containing various concentrations of VNP. The cells were stimulated for 3 min, and then the assay was terminated by aspirating the medium and adding 200 μL lysate (1% TritonX-100, 0.1 M HCl). The cGMP expression level was assessed with a cGMP ELISA kit and the data were analyzed with GraphPad prism 5.0. Graphs were generated from three separate assays; each point was assayed in triplicate.

2.5. Statistical analysis

The life science molecular design solutions software suite of Discovery Studio (version 3.5, Accelrys Inc., San Diego, CA) was

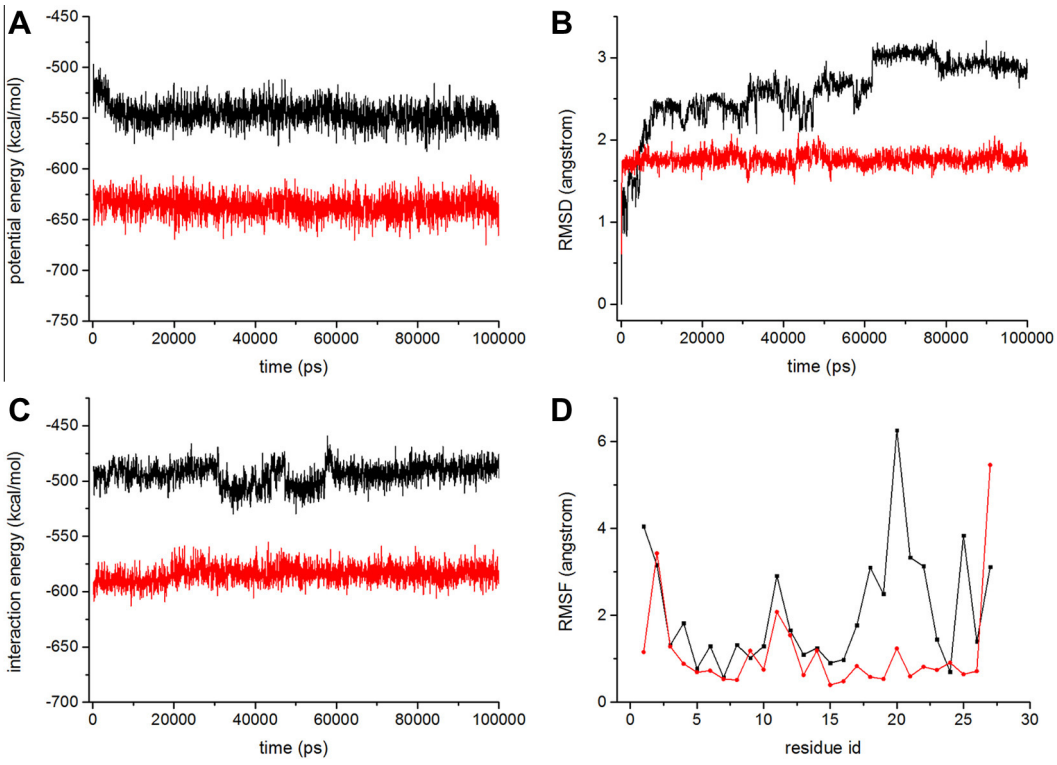

Fig. 1. Molecular dynamics of VNP with NPRA and NPRB. (A) Potential energy. (B) RMSD. (C) Interaction energy. (D) RMSF. VNP binding with NPRA (black) and with NPRB (red) was showed.

Table 1
Statistics for VNP with NPRA and NPRB.*

Contribution	VNP (NPRA)	VNP (NPRB)	Comparison (%)
Potential energy (kcal/mol)	−545.62 ± 11.33	−636.63 ± 10.32	−16.41
RMSD (Å)	2.61 ± 0.39	1.76 ± 0.08	−32.57
H-bonds (pairs)	28 ± 2	35 ± 2	25
Interaction energy (kcal/mol)	−494.28 ± 9.90	−584.35 ± 7.98	−18.82
Van der Waals (kcal/mol)	−132.48 ± 8.26	−172.22 ± 6.37	−30.00
Electrostatic (kcal/mol)	−361.79 ± 11.30	−412.02 ± 9.85	−13.88

* Values of each contribution were sampled and analyzed from 60 ns to 100 ns of the MD trajectory.

used for protein design and molecular dynamics simulation. Analysis of the MD data was done using Origin Pro (version 9.0). Mean ± standard error of mean (SEM) of the data were computed. The experimental data of bioactivity were analyzed using GraphPad Prism (version 5.0).

3. Results

3.1. Evaluation of the conformational sampling in MD simulation

VNP-binding simulation analysis showed that NPRB/VNP quickly achieved equilibration compared with the NPRA/VNP system (Fig. 1A). There was an 18.82% decrease in interaction energy with NPRB (−584.35 kcal/mol) than with NPRA (−494.28 kcal/mol) (Fig. 1C, Table 1). The RMSF variation trend of VNP was similar in both complexes; however, the values of SER1, Leu11, Ser18, GLY20 and Phe25 of VNP in NPRA/VNP were higher than in NPRB/VNP (Fig. 1D), which indicated that the VNP in NPRA/VNP adopted a much more flexible structure. While VNP in NPRB/VNP showed a stable structure with much lower RMSF values of most amino acid residues except for LEU2 and TYR27 on the end of each terminal.

The potential energy, RMSD values, Van der Waals and electrostatic energy of NPRB/VNP decreased by 16.41%, 32.57%, 30% and 13.88%, respectively, compared with NPRA/VNP (Table 1). In addition, 35 pairs H-bonds formed between VNP and NPRB, ~25% more than VNP and NPRA. The interaction energy between VNP and NPRB was −584.35 kcal/mol, which was 18.82% less than VNP and NPRA. These results showed a higher binding affinity between VNP and NPRB than between VNP and NPRA.

3.2. Configuration and binding interface analysis of VNP in NPRA and NPRB

We obtained similar NPRA/VNP and NPRB/VNP complex structures (Fig. 2A and B) in which VNP bound to the receptor by its ring structure, with each face of the ring interacted with a monomer of both receptors. However, the NPRB/VNP tail interaction interface was more hydrophobic than NPRA/VNP complex, which indicated that VNP bound more tightly to NPRB (Fig. 2C–F).

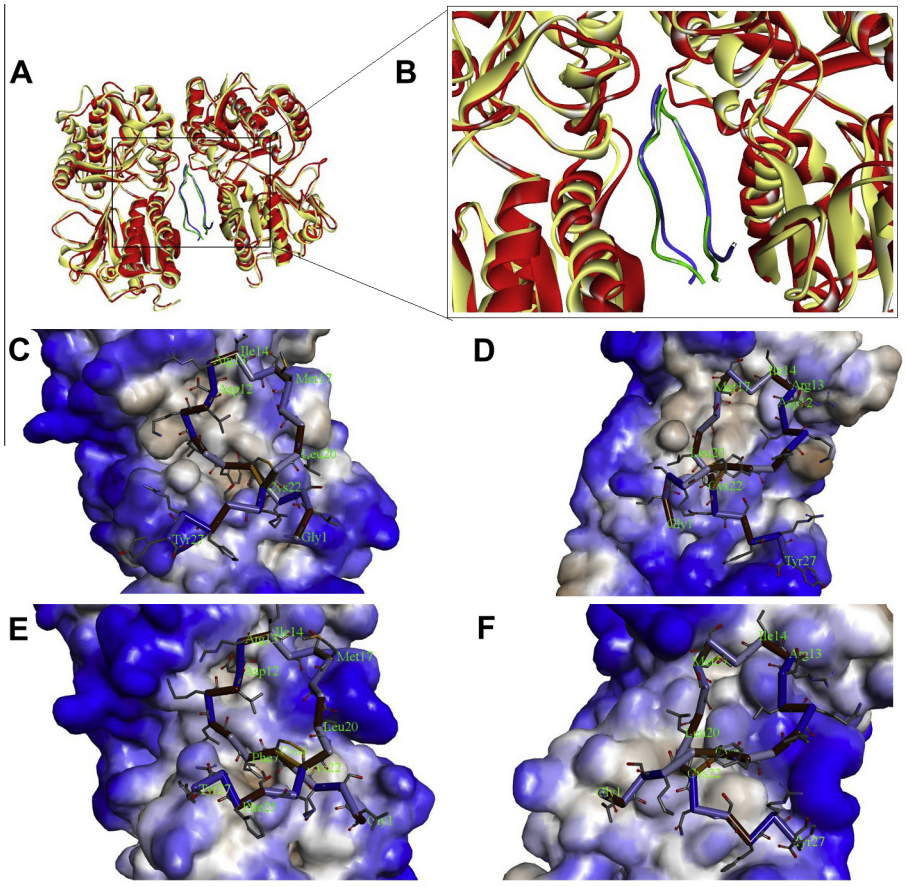

Fig. 2. Binding configuration of VNP with NPRA and NPRB. (A,B) Overlap of NPRA/VNP (yellow/green) and NPRB/VNP (red/blue). (C) Monomer A of NPRA. (D) Monomer B of NPRA. (E) Monomer A of NPRB. (F) Monomer B of NPRB. Monomers and VNP were colored by their hydrophobicity. Green label indicate conserved amino-acid residues of the VNP ring domain.

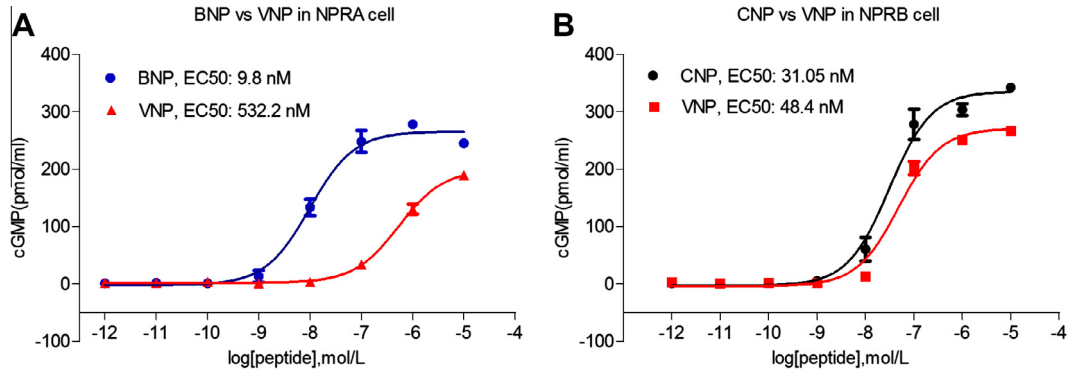

Fig. 3. cGMP stimulation by of NPRA and NPRB. HEK-293 cells stably expressing human NPRA were incubated with (A) BNP and VNP or (B) CNP and VNP for 3 min, and then intracellular cGMP concentrations were determined using a cGMP ELISA kit. The results are the means of three separate experiments conducted in triplicate ±S.E. ($n = 3$; p value was >0.05).

3.3. VNP bioactivity to activate NPRA and NPRB

To determine the ability of VNP to activate functional human NPRs (NPRA and NPRB), we stably expressed each individual receptor in wild-type HEK-293 cells devoid of endogenous NPRs. Using these cells, we could be confident that any increase in natriuretic peptide-dependent cGMP concentrations would result from the activation of a single stably-expressed receptor type. We assessed the ability of VNP to activate NPRA and NPRB (Fig. 3), while BNP and CNP natural ligands were used as positive controls against NPRA and NPRB. After 3 min of incubation, we determined the cGMP concentrations with a cGMP ELISA kit. Computed VNP in NPRA/NPRB activation analysis demonstrated higher by NPRB (EC_{50}, 48.4 nM) than by NPRA (EC_{50}, 532.2 nM).

4. Discussion

Conflicting results on the responses of receptor subtype of VNP have been reported. Muller and colleagues suggested that the action of VNP on NPRB in neurons occurs in fetal mice [20] while others researchers found that it had a significant effect on NPRA [21,22]. However there are no reports to demonstrate or confirm the binding receptor of VNP.

We measured the VNP/NPRA and VNP/NPRB-dependent cyclase activity in HEK-293 cells and found that VNP stimulated both NPRA and NPRB. However, the VNP-stimulated cGMP was 11-fold greater in NPRB than NPRA. Although VNP simulated less cGMP than the natural ligands of NPRA and NPRB (Fig. 3), the advantage of this peptide lies in its ability to bind both receptors. Molecular dynamics simulation results showed that the VNP/NPRB adopted a more stable conformation and formed 8 more pairs of H-bond more than VNP/NPRA. The two termini of VNP adopted a cross conformation, which gave the peptide a tighter volume and the VNP tails appeared like an overpass rather than a parallel and flat shape. This conformation vertically broadened the VNP within the ring structure suggesting that this aids the VNP tails to interact with more residues from both receptor monomers.

CD-NP, a chimera peptide composed of CNP fused to the C-terminal tail of Dendroaspis natriuretic peptide (DNP), where the DNP tail converted CNP from an NPRA non-agonist to a partial agonist while maintaining its ability to activate NPRB [19]. Another chimera AC-NP, also stimulates both NPRA and NPRB [23]. In this study, we confirmed that the ANP tail has a striking effect on CNP, converting it into a bi-functional agonist of both NPRA and NPRB. These data showed that drugs designed to activate NPRB alone, when combined with NPR-A, may provide more effective cardiac therapy for congestive heart failure than the FDA approved drug nesiritide (rhBNP), which only activates NPRA [24]. We therefore strongly suggest that VNP and other bi-functional chimera peptides represent a new generation of natriuretic peptide therapeutics.

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References

[1] G. Boerrigter, L.C. Costello-Boerrigter, J.C. Burnett Jr., Natriuretic peptides in the diagnosis and management of chronic heart failure, Heart Fail. Clin. 5 (2009) 501–514.
[2] T.G. Flynn, M.L. de Bold, A.J. de Bold, The amino acid sequence of an atrial peptide with potent diuretic and natriuretic properties, Biochem. Biophys. Res. Commun. 117 (1983) 859–865.
[3] T. Sudoh, K. Kangawa, N. Minamino, H. Matsuo, A new natriuretic peptide in porcine brain, Nature 332 (1988) 78–81.
[4] T. Sudoh, N. Minamino, K. Kangawa, H. Matsuo, C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain, Biochem. Biophys. Res. Commun. 168 (1990) 863–870.
[5] L.R. Potter, A.R. Yoder, D.R. Flora, L.K. Antos, D.M. Dickey, Natriuretic peptides: their structures, receptors, physiologic functions and therapeutic applications, Handb. Exp. Pharmacol. (2009) 341–366.
[6] Y. Cho, B.G. Somer, A. Amatya, Natriuretic peptides and their therapeutic potential, Heart Dis. 1 (1999) 305–328.
[7] C.M. Wei, C.H. Kim, V.M. Miller, J.C. Burnett Jr., Vasonatrin peptide: a unique synthetic natriuretic and vasorelaxing peptide, J. Clin. Invest. 92 (1993) 2048–2052.
[8] H.S. Feng, Y.M. Zang, M.Z. Zhu, J.M. Pei, Y.M. Wang, L. Wang, P.T. Shi, Comparison of vasorelaxing actions of vasonatrin peptide, C-type natriuretic peptide and atrial natriuretic peptide, Sheng Li Xue Bao 51 (1999) 515–520.
[9] M.Q. Dong, M.Z. Zhu, J. Yu, L.J. Shang, H.S. Feng, Comparison of inhibitory effects of three natriuretic peptides on the proliferation of pulmonary artery smooth muscle cells of rats, Sheng Li Xue Bao 52 (2000) 252–254.
[10] S.Y. Lu, M.Z. Zhu, D.S. Wang, S.Y. Chen, W.D. Zhang, H. Dong, J. Yu, H.T. Guo, Inhibition of the proliferation of smooth muscle cells from human coronary bypass vessels by vasonatrin peptide, Physiol. Res. 53 (2004) 387–393.
[11] S.Y. Lu, M.Z. Zhu, H.T. Guo, J. Yu, Q.M. Wei, Inhibition of moderate hypoxia-induced protein synthesis by vasonatrin peptide in cultured neonatal rat cardiomyocytes, Sheng Li Xue Bao 54 (2002) 7–11.
[12] S.Y. Lu, D.S. Wang, M.Z. Zhu, Q.H. Zhang, Y.Z. Hu, J.M. Pei, Inhibition of hypoxia-induced proliferation and collagen synthesis by vasonatrin peptide in cultured rat pulmonary artery smooth muscle cells, Life Sci. 77 (2005) 28–38.
[13] Y. Morishita, T. Sano, K. Ando, Y. Saitoh, H. Kase, K. Yamada, Y. Matsuda, Microbial polysaccharide, HS-142-1, competitively and selectively inhibits ANP binding to its guanylyl cyclase-containing receptor, Biochem. Biophys. Res. Commun. 176 (1991) 949–957.
[14] L.R. Potter, S. Abbey-Hosch, D.M. Dickey, Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions, Endocr. Rev. 27 (2006) 47–72.
[15] M.B. Anand-Srivastava, Natriuretic peptide receptor-C signaling and regulation, Peptides 26 (2005) 1044–1059.

[16] G.E. Woodard, X. Li, J. Brown, J.A. Rosado, Receptor subtypes for vasonatrin peptide in renal glomeruli and arteries, Regul. Pept. 129 (2005) 183–189.
[17] B.Y. Chen, J.K. Chen, M.Z. Zhu, D.L. Zhang, J.S. Sun, J.M. Pei, H.S. Feng, X.X. Zhu, J. Jin, J. Yu, AC-NP: a novel chimeric peptide with natriuretic and vasorelaxing actions, PLoS One 6 (2011) e20477.
[18] O. Lisy, J.C. Burnett, The design, synthesis and cardiorenal actions of a new chimeric natriuretic peptide CD-NP, J. Am. Coll. Cardiol. 41 (2003). 312a–312a.
[19] D.M. Dickey, J.C. Burnett Jr., L.R. Potter, Novel bifunctional natriuretic peptides as potential therapeutics, J. Biol. Chem. 283 (2008) 35003–35009.
[20] D. Muller, B. Hida, G. Guidone, R.C. Speth, T.V. Michurina, G. Enikolopov, R. Middendorff, Expression of guanylyl cyclase (GC)-A and GC-B during brain development: evidence for a role of GC-B in perinatal neurogenesis, Endocrinology 150 (2009) 5520–5529.
[21] B.Y. Chen, P. Qu, R. Tie, M.Z. Zhu, X.X. Zhu, J. Yu, Protecting effects of vasonatrin peptide against carbon tetrachloride-induced liver fibrosis, Regul. Pept. 164 (2010) 139–143.
[22] B.Y. Chen, R. Tie, P. Qu, M.Z. Zhu, X.X. Zhu, J. Jin, J. Yu, Vasonatrin peptide, a new regulator of adiponectin and interleukin-6 production in adipocytes, J. Endocrinol. Invest. 34 (2011) 742–746.
[23] X. Zhu, Y. Wang, A. Schwiebs, T. Walther, Chimeric natriuretic peptide ACNP stimulates both natriuretic peptide receptors, the NPRA and NPRB, Mol. Cell. Endocrinol. 366 (2013) 117–123.
[24] D.M. Dickey, D.R. Flora, P.M. Bryan, X. Xu, Y. Chen, L.R. Potter, Differential regulation of membrane guanylyl cyclases in congestive heart failure: natriuretic peptide receptor (NPR)-B, not NPR-A, is the predominant natriuretic peptide receptor in the failing heart, Endocrinology 148 (2007) 3518–3522.