Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 358 (2007) 145–149
BBRC
www.elsevier.com/locate/ybbrc

Dendroaspis natriuretic peptide binds to the natriuretic peptide clearance receptor

Douglas G. Johns [a,*], Zhaohui Ao [a], Bradley J. Heidrich [a], Gerald E. Hunsberger [b], Taylor Graham [c], Lisa Payne [c], Nabil Elshourbagy [c], Quinn Lu [c], Nambi Aiyar [a], Stephen A. Douglas [a]

[a] *Vascular Biology and Thrombosis Department, Cardiovascular and Urogenital Center for Excellence in Drug Discovery, GlaxoSmithKline, King of Prussia, PA 19406, USA*
[b] *Respiratory and Inflammatory Diseases Center for Excellence in Drug Discovery, GlaxoSmithKline, King of Prussia, PA 19406, USA*
[c] *Gene Expression and Protein Biochemistry, GlaxoSmithKline, King of Prussia, PA 19406, USA*

Received 11 April 2007
Available online 19 April 2007

Abstract

Dendroaspis natriuretic peptide (DNP) is a newly-described natriuretic peptide which lowers blood pressure via vasodilation. The natriuretic peptide clearance receptor (NPR-C) removes natriuretic peptides from the circulation, but whether DNP interacts with human NPR-C directly is unknown. The purpose of this study was to test the hypothesis that DNP binds to NPR-C. ANP, BNP, CNP, and the NPR-C ligands AP-811 and cANP(4–23) displaced [^{125}I]-ANP from NPR-C with pM-to-nM K_i values. DNP displaced [^{125}I]-ANP from NPR-C with nM potency, which represents the first direct demonstration of binding of DNP to human NPR-C. DNP showed high pM affinity for the GC-A receptor and no affinity for GC-B ($K_i > 1000$ nM). DNP was nearly 10-fold more potent than ANP at stimulating cGMP production in GC-A expressing cells. Blockade of NPR-C might represent a novel therapeutic approach in augmenting the known beneficial actions of DNP in cardiovascular diseases such as hypertension and heart failure.

Keywords: Natriuretic peptides; Natriuretic peptide clearance receptor; NPR-C; Heart failure; DNP, *Dendroaspis* natriuretic peptide

Dendroaspis natriuretic peptide (DNP), originally isolated from green mamba snake (*Dendroaspis angusticeps*) venom [1], is a newly-described member of the natriuretic peptide family, which includes atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). Reports of plasma and tissue immunoreactivity for DNP in humans and rats [2–6], has raised the possibility that DNP could be another endogenous natriuretic peptide, though this is currently under debate [7], as a gene which encodes for mammalian DNP has not been reported. However, DNP has been shown to possess vasodilator, natriuretic and diuretic properties in animal models and human tissues [5,8,9], similar to ANP, BNP, and CNP [10,11].

The vasodilator and natriuretic/diuretic effects of the known endogenous mammalian natriuretic peptides are mediated by guanylyl-cyclase-coupled receptors GC-A (for ANP and BNP) and GC-B (for CNP) [12,13]. Clearance of ANP, BNP, and CNP is thought to occur primarily via either enzymatic degradation by neutral endopeptidase or by internalization of the peptides by the natriuretic peptide clearance receptor (NPR-C). The observation that DNP is resistant to NEP degradation [14] implies that the primary mechanism for clearance of DNP from the circulation is via another mechanism, possibly NPR-C. Although Schweitz et al. [1] reported that

* Corresponding author. Fax: +1 610 270 6201.
E-mail address: Douglas.G.Johns@gsk.com (D.G. Johns).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.04.079

DNP binds to vascular smooth muscle cells and inferred that this was non-GC-coupled receptor, a direct binding interaction between DNP and NPR-C has not been characterized.

The purpose of this study was to test the hypothesis that DNP binds to NPR-C, thus elaborating a role of NPR-C as a potential clearance route for this newly-described natriuretic peptide. The present report demonstrates that DNP does bind to human recombinant NPR-C with characteristics similar to other natriuretic peptides, and verifies the potency and activity of DNP against the GC-A receptor.

Methods

Materials. Natriuretic peptides [ANP, BNP, CNP, DNP, cANP(4–23)] were purchased from Bachem Bioscience, Inc. (King of Prussia, PA). AP-811 [2-naphtoyl-236-Arg-Ile-Asp-Arg-(S)-(−)-2-methyl-butylamide] was purchased from California Peptide Research, Inc. (Napa, CA). [^{125}I]-ANP(3–28) and [^{125}I]-CNP were purchased from Amersham Biosciences (Piscataway, NJ). Chinese hamster ovary (CHOK1) cells were obtained from American Tissue Type Collection (Manassas, VA) and maintained in Dulbecco's modified essential medium containing 15% fetal bovine serum.

Expression of recombinant human NPR-C, GC-A, and GC-B. cDNA sequences for human NPR-C (GenBank AF022998), GC-A (GenBank NM_000906), and GC-B (GenBank NM_003995) were PCR amplified from a human kidney Marathon library (Clontech, Mountain View, CA) and subcloned into the pcDNA3.2/GW/D-TOPO vector via TOPO cloning (Invitrogen, Carlsbad, CA). BacMam viruses for the genes were generated using the BacMam transfer vectors and protocols as described previously [15]. The BacMam transfer vector for NPR-C was generated by PCR amplification with codons for 6 × His added at the 3′ end of the open reading frame, and subcloned into the *Eco*RI/*Hpa*I sites of pFastNot-CMV. The BacMam transfer vector for GC-A was generated by inserting a *Spe*I/*Xba*I fragment containing the ORF into the XbaI site of pFast-Bacmam-NA. The BacMam transfer vector for GC-B was generated by inserting a *Not*I/*Asc*I fragment containing the ORF into the *Not*I/*Asc*I sites of pFastBacmam1. For expression of the natriuretic peptide receptors, CHOK1 cells were grown to 90% confluence in DMEM/F12 medium with 10% FBS, and transduced with a BacMam virus (MOI = 5). After 48 h, sodium butyrate was added (2 mmol l^{-1}) and the cells were further incubated for another 24 h.

Radioligand binding. Membranes were prepared from CHO-K1 cells scraped in 1 mmol l^{-1} EDTA in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline. Cells were washed by centrifugation and resuspended in ice-cold buffer (10 mmol l^{-1} Tris–HCl [pH 7.4], 5 mmol l^{-1} Na EDTA, 0.1 mmol l^{-1} phenylmethylsulfonyl fluoride, 1.0 mg ml^{-1} bacitracin, 1.0 mg ml^{-1} aprotinin). Following homogenization (Dounce homogenizer; Bellco Glass, Inc., Vineland, NJ, USA) and centrifugation (47,000*g*, 20 min, 4 °C), the resultant pellets were washed twice by centrifugation (in 25 mmol l^{-1} Tris–HCl [pH 7.4], 5 mmol l^{-1}$MgCl_2$, 2 mmol l^{-1} EGTA, 0.1 mg ml^{-1} bacitracin) and resuspended (5 mg ml^{-1}) for storage (−70 °C). Protein concentration was measured by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA).

Specific binding of [^{125}I]-ANP(3–28) was determined using crude membrane preparations of cells expressing recombinant human GC-A or NPR-C receptors. For human recombinant GC-B receptor binding, [^{125}I]-CNP was used. Membranes were incubated for 60 min at 25 °C in 200 μl buffer [25 mmol l^{-1} Tris–HCl, pH 7.4, 5 mmol l^{-1} $MgCl_2$, and 0.1% BSA] containing 30 pmol l^{-1} [^{125}I]-ANP(3–28), which was determined in pilot experiments to show identical binding characteristics as full-length [^{125}I]-ANP(1–28), or 100 pmol l^{-1} [^{125}I]-CNP. In competition binding studies membranes (30–50 μg of membrane protein ml^{-1}) were incubated with increasing concentrations (1 pmol l^{-1}–1 μmol l^{-1}) of competing ligands and [^{125}I]-ANP(3–28) or [^{125}I]-CNP for 60 min at 25 °C. The incubation was terminated by the addition of 2 ml of ice-cold wash buffer (0.9% NaCl) followed by rapid filtration over Whatman GF/C filters, which were washed three times with 4 ml of ice-cold wash buffer. For experiments using [^{125}I]-ANP(3–28) as the radiolabel, non-specific binding was determined in the presence of 1 μmol l^{-1} unlabeled ANP(3–28). For experiments using [125I]-CNP, non-specific binding was determined in the presence of 1 μmol l^{-1} unlabeled CNP. Filters were counted in a gamma counter.

cGMP formation. Formation of cGMP in recombinant receptor-expressing cells was measured using a commercially available fluorescence detection kit (HitHunter™, DiscoveRx, Fremont, CA). Briefly, CHO-K1 cells expressing human recombinant GC-A or GC-B were harvested from cell culture media by low-speed centrifugation and resuspended in phosphate buffered saline containing 1 mmol l^{-1} 3-isobutyl-1-methylxanthine. Cells were distributed into 96-well assay plates at a density of 150,000 cells well^{-1}. Natriuretic peptides were added to each well at increasing concentrations to generate a concentration response curve. Cyclic GMP was detected following lysis of cells using a polyclonal anti-cGMP antibody and enzyme-based chemiluminescence per the manufacturer's instructions. Concentration of cGMP was determined using a standard curve.

Data analysis. Values are expressed as mean ± standard error of three independent experiments performed with duplicate replicates. Non-linear curve-fitting (GraphPad Prism, San Diego, CA, USA) was used to calculate equilibrium binding affinity (K_d) and maximum binding site density (B_{max}) from saturation-binding experiments in addition to determining competitor affinity constant (K_i) from competition binding experiments and agonist potency (EC_{50}) and efficacy (R_{max}) from cGMP formation assays.

Results

Radioligand binding

Because NPR-C is known to bind multiple natriuretic peptides, both [^{125}I]-ANP(3–28) and [^{125}I]-CNP were used for saturation binding experiments with human recombinant NPR-C to verify the pharmacological profile of BacMam-expressed receptor. In these experiments, [^{125}I]-ANP(3–28) showed saturable and specific binding for the recombinant NPR-C receptor with a K_d and B_{max} of approximately 200 pmol l^{-1} and 3.5 pmol mg^{-1} protein, respectively (Table 1). [^{125}I]-CNP showed saturable and specific binding at recombinant NPR-C with a K_d and B_{max} of approximately 130 pmol mg^{-1} and 700 fmol mg^{-1} protein, respectively (Table 1). For the natriuretic receptors GC-A and GC-B, each radioligand showed characteristic affinities for its respective receptor ([^{125}I]-ANP(3–28) for GC-A and [^{125}I]-CNP for GC-B). A summary of K_d and B_{max} values for all recombinant natriuretic peptide receptors is shown in Table 1.

For competition experiments, [^{125}I]-ANP(3–28) was used for NPR-C and GC-A, and [^{125}I]-CNP was used for

Table 1
Saturation binding of recombinant natriuretic peptide receptors

Receptor (ligand)	K_d (pmol l^{-1})	B_{max} (fmol/mg^{-1})
NPR-C ([^{125}I]-ANP(3–28))	205 ± 36	3478 ± 1,118
NPR-C ([^{125}I]-CNP)	131 ± 147	710 ± 763
GC-A ([^{125}I]-ANP(3–28))	156 ± 74	1405 ± 1281
GC-B ([^{125}I]-CNP)	403 ± 112	2367 ± 952

Data are presented as means ± SEM of $n = 3$ independent experiments.

GC-B experiments. In membranes from CHO-K1 cells expressing human recombinant GC-A receptor, the endogenous natriuretic peptide ANP displaced bound radioligand with characteristic potency (Fig. 1), while GC-B-selective CNP and the NPR-C-selective ligand AP-811 [16] did not displace [^{125}I]-ANP(3–28) (Fig. 1). DNP displaced [^{125}I]-ANP(3–28) with a K_i of 100 pmol l^{-1} (Fig. 1). In membranes from CHO-K1 cells expressing human recombinant GC-B receptor, CNP displaced [^{125}I]-CNP with a K_i of 1 nmol l^{-1}, where DNP did not displace CNP (Fig. 1). In membranes from CHO-K1 cells expressing human recombinant NPR-C, DNP displaced radiolabeled ANP(3–28) from NPR-C expressing membranes with a K_i of 4 nmol l^{-1} (Fig. 2). The endogenous natriuretic peptides ANP, BNP, and CNP, and the NPR-C-selective ligands AP811 and cANP(4–23) all displaced [^{125}I]-ANP(3–28) with potencies ranging from 700 pmol l^{-1} to 7 nmol l^{-1}. A summary of the K_i values for all peptides tested are shown in Table 2.

cGMP formation

DNP stimulated cGMP formation in GC-A-expressing cells with an effect that was approximately 10-fold more potent than ANP (though this was not statistically significant; Fig. 1). DNP had no effect on cGMP formation in cells expressing recombinant GC-B.

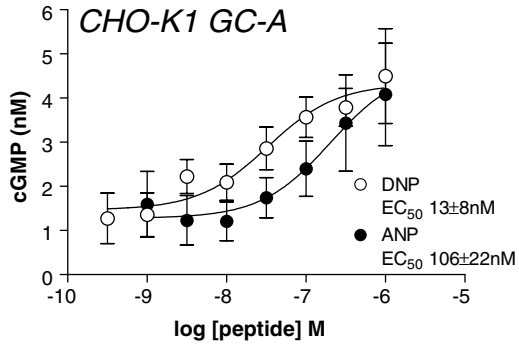

Fig. 2. DNP stimulates cGMP in cells expressing human recombinant GC-A receptor. CHO-K1 cells expressing human recombinant GC-A were treated with increasing concentrations of either DNP or ANP and cGMP was measured as described in Methods.

Discussion

The purpose of the present study was to determine whether DNP does, in fact, interact with NPR-C. The reported resistance of DNP to degradation by neutral endopeptidase [14] leaves the clearance receptor NPR-C as the likely route of removal of DNP from the circulation. In the original description of DNP as a vasodilatory natriuretic peptide by Schweitz et al. [1], DNP displaced radiolabeled ANP from rat aortic smooth muscle cells. Because aortic smooth muscle cells express NPR-C, this was indi-

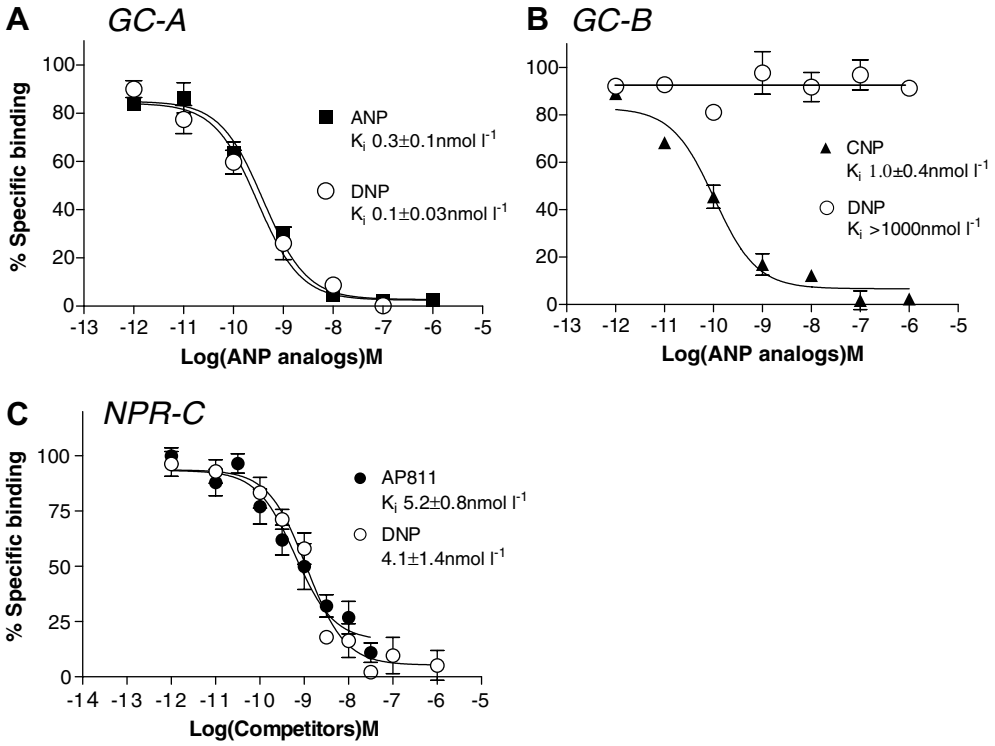

Fig. 1. DNP displaces radiolabeled natriuretic peptides from GC-A and NPR-C. (A) Displacement of [^{125}I]-ANP(3–28) from human recombinant GC-A by ANP and DNP, (B) displacement of [125I]-CNP from human recombinant GC-B by CNP but not DNP, and (C) displacement of [^{125}I]-ANP(3–28) from human recombinant NPR-C by the NPR-C-selective ligand AP-811 and DNP.

Table 2
K_i, values (nmol l^{-1}) for various natriuretic peptides at each recombinant natriuretic peptide receptor

	GC-A[a]	GC-B[b]	NPR-C
ANP	0.3 ± 0.1	15.1 ± 5.5	0.8 ± 0.2
BNP	1.8 ± 0.8	9.8 ± 2.2	2.5 ± 0.4
CNP	>1000	1.0 ± 0.4	7.2 ± 0.8
ANP(3–28)	0.1 ± 0.01	2.5 ± 0.8	0.7 ± 0.3
CNP(4–23)	>1000	—	0.9 ± 0.3
AP811	>1000	>1000	5.2 ± 0.8
DNP	0.1 ± 0.03	>1000	4.1 ± 1.4

Data are presented as means ± SEM of $n = 3$ independent experiments.
[a] GC-A and NPR-C data were generated using [^{125}I]-ANP(3–28).
[b] GC-B data were generated using [^{125}I]-CNP.

rect evidence that DNP interacted with the clearance receptor. However, a specific binding interaction between DNP and NPR-C and pharmacological characterization of this interaction has never been reported. In the current study, human recombinant natriuretic peptide receptors were used for characterization of ligand binding. This study represents the first report of the BacMam system as a method for expression of these receptors in mammalian cell culture systems. For this reason, each receptor was characterized using classical natriuretic peptides and peptide analogs using radioligand binding. For the GC-A and NPR-C receptors, [^{125}I]-ANP(3–28) showed saturation kinetics with K_d values in accordance with previously published results where [^{125}I]-ANP was used [13]. Furthermore, the potencies of various receptor ligands (e.g. ANP, BNP for GC-A; ANP, BNP, CNP, AP-811, cANP(4–23) for NPR-C) were in line with published data [13,17] reinforcing use of this system to overexpress each of these receptors. The potency of DNP for increasing cGMP (13 nmol l^{-1}) was approximately 10-fold more potent than ANP (106 nmol l^{-1}) in cells expressing human recombinant GC-A, but within the range of potencies reported by others in endogenous cells [1]. This verifies activity of DNP against GC-A and validates the use of this recombinant receptor as a comparative tool.

The K_i of DNP (approximately 100 pmol l^{-1}) for GC-A was similar to that of ANP (approximately 300 pmol l^{-1}), a natural endogenous ligand for this receptor. The affinity of DNP for NPR-C was about 30-fold lower than for GC-A, implying some degree of selectivity of DNP for the GC-A receptor. Further, DNP was about 30-fold less potent at displacing radiolabeled ANP from NPR-C compared to ANP and BNP. This suggests that while DNP does interact with the NPR-C receptor, it might not be cleared as effectively from the circulation as other natriuretic peptides, which possess a greater affinity. This, coupled with resistance to neutral endopeptidase degradation, implies that exogenous DNP administration could represent a more effective natriuretic peptide therapy than previously reported approaches (e.g. recombinant BNP) for congestive heart failure. However, this does not rule out the possible contribution for other as-yet unidentified catabolic/clearance routes for DNP.

The relevance of the current findings to the clearance of endogenous DNP remains to be determined, as definitive evidence that a human isoform of DNP exists is lacking. Reports of "DNP-like" immunoreactivity describe levels of DNP ranging from 1 to 50 pmol l^{-1} in the plasma of normal subjects [2,3,18] as well as in various tissues [5,6]. While the plasma values are 20- to 1000-fold lower than the affinity of DNP for NPR-C reported in the current study, further investigation and additional tools are required to definitively demonstrate the existence of human endogenous DNP.

The data from the current study describe, for the first time, the direct pharmacological interaction of DNP with NPR-C. Consideration of DNP administration as a therapy for cardiovascular diseases such as congestive heart failure could be strengthened with the notion that DNP is cleared from the circulation via NPR-C. Taken further, blockade of NPR-C could represent a novel therapeutic strategy in which levels of natriuretic peptides, including exogenous DNP, are maintained to offer compensatory protection in cases of cardiovascular diseases such as heart failure and hypertension.

Conflict of interest statement

All authors on the current manuscript are employed by Glaxosmithkline.

References

[1] H. Schweitz, P. Vigne, D. Moinier, C. Frelin, M. Lazdunski, A new member of the natriuretic peptide family is present in the venom of the green mamba (*Dendroaspis angusticeps*), J. Biol. Chem. 267 (1992) 13928–13932.
[2] J. Schirger, D. Heublein, H. Chen, O. Lisy, M. Jougasaki, P. Wennberg, J. Burnett, Presence of *Dendroaspis* natriuretic peptide-like immunoreactivity in human plasma and its increase during human heart failure, Mayo Clin. Proc. 74 (1999) 126–130.
[3] G.E. Woodard, J.A. Rosado, J. Brown, *Dendroaspis* natriuretic peptide-like immunoreactivity and its regulation in rat aortic vascular smooth muscle, Peptides 23 (2002) 23–29.
[4] S. Lee, S.K. Park, K.P. Kang, S.K. Kang, S.Z. Kim, W. Kim, Relationship of plasma *Dendroaspis* natriuretic peptide-like immunoreactivity and echocardiographic parameters in chronic haemodialysis patients, Nephrology (Carlton) 9 (2004) 171–175.
[5] O. Lisy, J.G. Lainchbury, H. Leskinen, J.C. Burnett Jr., Therapeutic actions of a new synthetic vasoactive and natriuretic peptide, *Dendroaspis* natriuretic peptide, in experimental severe congestive heart failure, Hypertension 37 (2001) 1089–1094.
[6] O. Lisy, M. Jougasaki, D.M. Heublein, J.A. Schirger, H.H. Chen, P.W. Wennberg, J.C. Burnett, Renal actions of synthetic *Dendroaspis* natriuretic peptide, Kidney Int. 56 (1999) 502–508.
[7] A.M. Richards, J.G. Lainchbury, M.G. Nicholls, A.V. Cameron, T.G. Yandle, *Dendroaspis* natriuretic peptide: endogenous or dubious? Lancet 359 (2002) 5–6.
[8] P.J.M. Best, J.C. Burnett Jr., S.H. Wilson, D.R. Holmes Jr., A. Lerman, *Dendroaspis* natriuretic peptide relaxes isolated human arteries and veins, Cardiovasc. Res. 53 (2002) 375–384.
[9] E. Collins, M.P. Bracamonte, J.C. Burnett Jr., V.M. Miller, Mechanism of relaxations to *Dendroaspis* natriuretic peptide in canine coronary arteries, J. Cardiovasc. Pharmacol. 35 (2000) 614–618.

[10] Y. Cho, B.G. Somer, A. Amatya, Natriuretic peptides and their therapeutic potential, Heart Disease 5 (1999) 305–328.
[11] J.C. Burnett Jr., Novel therapeutic directions for the natriuretic peptides in cardiovascular diseases: what's on the horizon, J. Cardiol. 48 (2006) 235–241.
[12] S. Shulz, S.A. Waldman, The guanylyl cyclase family of natriuretic peptide receptors, Vitam. Horm. 57 (1999) 123–151.
[13] K.J. Koller, D.V. Goeddel, Molecular biology of the natriuretic peptides and their receptors, Circulation 86 (1992) 1081–1088.
[14] H.H. Chen, J.G. Lainchbury, J.C. Burnett Jr., Natriuretic peptide receptors and neutral endopeptidase in mediating the renal actions of a new therapeutic synthetic natriuretic peptide *Dendroaspis* natriuretic peptide, J. Am. Coll. Cardiol. 40 (2002) 1186–1191.
[15] J.A. Fornwald, Q. Lu, D. Wang, R.S. Ames, Gene expression in mammalian cells using BacMam, a modified baculovirus system, in: D.W. Murhammer (Ed.), Methods in Molecular Biology, Baculovirus and Insect Cell Expression Protocols, Vol. 338, Humana Press, New Jersey, 2007, pp. 95–114.
[16] C.A. Veale, V.C. Alford, D. Aharony, D.L. Banville, R.A. Bialecki, F.J. Brown, J.R. Damewood Jr., C.L. Dantzman, P.D. Edwards, R.T. Jacobs, R.C. Mauger, M.M. Murphy, W. Palmer, K.K. Pine, W.L. Rumsey, L.E. Garcia-Davenport, A. Shaw, G.B. Steelman, J.M. Surian, E.P. Vacek, The discovery of non-basic atrial natriuretic peptide clearance receptor antagonists. Part 1, Bioorg. Med. Chem. Lett. 10 (2000) 1949–1952.
[17] S. Koyama, T. Inoue, T. Terai, K. Takimoto, M. Kato, K. Ito, M. Neya, J. Seki, Y. Kobayashi, Y. Kyogoku, K. Yoshida, AP-811, a novel ANP-C receptor selective agonist, Int. J. Peptide. Prot. Res. 43 (1994) 332–336.
[18] E. Fabrega, J. Crespo, M. Rivero, F. Casafont, B. Castro, M.T. Garcia-Unzueta, J.A. Amado, F. Pons-Romero, *Dendroaspis* natriuretic peptide in heptic cirrhosis, Am. J. Gastroenterol. 96 (2001) 2724–2729.