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A potent and selective natriuretic peptide receptor-3 blocker 11-mer peptide created by hybridization of musclin and atrial natriuretic peptide

Naoki Nishizawa, Goshi Nakamura, Yoko Noguchi, Hideyuki Nakagawa, Ayako Shimizu, Masaharu Nakayama, Shiro Takekawa, Taiji Asami *

Pharmaceutical Research Division, Takeda Pharmaceutical Company Ltd, Fujisawa 251-8555, Japan

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ABSTRACT

The natriuretic peptide (NP) system is a critical endocrine, autocrine, and paracrine system and has been investigated for potential use against cardiovascular and metabolic diseases. The clearance of NPs is regulated by the proteolysis of neutral endopeptidase (NEP) and by endocytosis via natriuretic peptide receptor-3 (NPR3). A linear NPR3-selective peptide, [Cha^{8}]-ANP(7-16)-NH_2 (**1**), showed potent binding affinity for NPR3 but poor predicted chemical stability due to its free thiol group. A 12-mer peptide (**9**) without a thiol group was designed by the hybridization of two NPR3-binding peptides: a linear ANP fragment peptide analog and musclin, a murine member of the bHLH family of transcription factors, possessed high binding affinity and strict selectivity for NPR3. To increase the proteolytic resistance of **9**, amino acid substitutions at the cleavage sites led to hydroxyacetyl-[D-Phe^{5},D-Hyp^{7},Cha^{8},D-Ser^{9},Hyp^{11},Arg(Me)^{14}]-ANP(5-15)-$NHCH_3$ (**23**), showing high and selective binding affinity for NPR3 over NPR1 and excellent stability in mouse serum. Compound **23** increased intracellular cGMP concentrations in primary cultured adipocytes, and continuous administration induced substantial plasma cGMP elevation in mice, suggesting its potential to clarify the physiological role of NPR3 and its therapeutic application.

Atrial natriuretic peptide or A-type natriuretic peptide (ANP) was isolated from the human atrium and identified as a 28-amino acid peptide.[1] ANP is synthesized and stored in the heart and is secreted into the blood in response to atrial expansion due to increased blood volume. ANP acts on the kidneys to promote sodium excretion.[2] The diuretic effect of ANP dilates blood vessels and lowers blood pressure.[3,4] Through these actions, ANP plays a central role in the regulation of blood circulation. In addition, ANP functions to suppress the repair and self-renewal of vascular wall cells.[5,6] ANP is involved in the physiological formation, maintenance, and adaptation of the cardiovascular system. Mice deficient in ANP receptor genes show salt-dependent hypertension.[7] A frameshift mutation in ANP gene was identified in patients with familial atrial fibrillation,[8] and its frameshift product, called mANP,[9] exhibited increased protection from proteolytic degradation and a markedly greater half-life than that of ANP.[10] Deficiency of the ANP receptor gene causes essential hypertension, cardiac hypertrophy, and heart failure, indicating that abnormalities in ANP and its receptor are involved in pathogenesis of the cardiovascular system.[7]

After 30 min of continuous intravenous administration of human ANP (hANP), cardiac function-improving effects were observed in patients with congestive heart failure.[11] hANP significantly reduced systolic blood, pulmonary artery, pulmonary artery wedge, and right atrial pressures 30 min after the start of administration, concomitant with significant improvement in cardiac index and cardiac output. On the basis of these outstanding clinical results, the use of hANP (carperitide) was approved in 1995 in Japan. After more than 20 years, it has been widely applied clinically as a first-line treatment for acute heart failure. In recent years, the deterioration and acute exacerbation of heart failure has been understood to be caused by increased neurohumoral factors, and increasing attention is being paid to the heart-protective effects of ANP. It turns out that ANP inhibits cardiac hypertrophy and

Abbreviations: ANP, atrial natriuretic peptide; Arg(Me), N^{ω}-methylarginine; cGMP, cyclic guanosine monophosphate; Cha, 3-cyclohexylalanine; GC, guanylate cyclase; hANP, human ANP; Hyp, *trans*-4-hydroxyproline; LC/MS/MS, liquid chromatography–tandem mass spectrometry; mANP, mutant ANP; NPR3, natriuretic peptide receptor-3; PBS, phosphate buffered saline.

* Corresponding author.
E-mail address: taiji.asami@takeda.com (T. Asami).

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fibrosis not only as a circulating hormone but also as a local cardiac factor.

Obesity has attracted much attention as a basic pathological condition of arteriosclerotic diseases. It has been previously reported that ANP promotes lipolysis in human adipocytes.[12] More recent studies have revealed that ANP changes the phenotype of white adipose tissue to a brown adipose tissue-like phenotype.[13] Cardiovascular disease develops on the basis of conditions such as high blood pressure, obesity, and impaired glucose tolerance. These findings suggest that ANP may be a treatment option not only for diseases such as myocardial infarction and heart failure but also for their risk factors. Inhibitors of neutral endopeptidase 24.11, an ANP-degrading enzyme, are being developed for clinical application. Increasing the amount of endogenous ANP may be a potential therapy not only for hypertension and chronic heart failure but also for peripheral arterial obstructive disease, pulmonary arterial hypertension, and impaired glucose tolerance with obesity.

The ANP receptor, known as natriuretic peptide receptor A/guanylate cyclase A/atrionatriuretic peptide receptor A and abbreviated as NPR1 (ANPRA, NPRA), is responsible for the hormonal activity of both ANP and its related peptide, brain natriuretic peptide or B-type natriuretic peptide (BNP). These molecules increase cyclic guanosine monophosphate (cGMP) levels via activation of guanylate cyclase (GC) in the intracellular domain of NPR1. The increased intracellular cGMP concentrations lead to the activation of cGMP-dependent protein kinases, which then induce multiple cellular functions. Subcutaneous administration of hANP was reported to increase plasma cGMP levels concomitant with the increased intracellular cGMP concentration in humans,[14] suggesting that elevated plasma cGMP is a useful biomarker of the induction of ANP signals. Conversely, natriuretic peptide receptor C (NPR3, ANPRC, NPRC) does not have a GC domain and is not associated with any of the known physiological functions of diuretic peptides.[15] NPR3 acts as a clearance receptor of diuretic peptides and strongly binds to ANP and its related peptides to remove them from the blood.[16] Therefore, blockers of NPR3 would also have the potential to improve the hormonal activity of ANP. However, no NPR3 blockers have been reported for in vivo use.

C-ANP$_{4-23}$ (Arg-Ser-Ser-Cys*-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Cys*, where Cys* indicates a disulfide bridge) is a well-known cyclic peptide that is an NPR3 blocker (Table 1),[16–18] though its stability was found to be poor in mouse serum (Table 2). The residual ratio was 4.0% after incubation in 10% mouse serum/phosphate-buffered saline (PBS) for 30 min. A linear peptide analog of C-ANP$_{4-23}$ (free Cys form) showed potent binding affinity for NPR3 (IC_{50}: 0.65 nM), comparable with that of C-ANP$_{4-23}$. A shorter analog of the linear peptide analog of C-ANP$_{4-23}$ (**1**) composed of ten amino acid residues, including Cha[8], maintained its moderate binding affinity with an IC_{50} value of 1.5 nM (Table 1). Compound **1** seemed to be a good lead compound for the discovery of biologically stable NPR3 blockers; however, the Cys residue at position 7 in **1** raised a concern due to its poor chemical stability. An *N*-terminal acetylation (**2**), Ala (**4**) or Ser (**3**) substitution at position 7, and deletion of Cys[7] (**5**, **6**) attenuated the binding affinity for NPR3 by about 10 times compared with that of **1**. It is conceivable that the *N*-terminal amino and free thiol groups are involved in receptor binding.

Musclin is a skeletal muscle-derived secretory factor that has been characterized as a stimulator that induces insulin resistance in mice.[19] Musclin is a 101-mer NPR3 inhibitory peptide that binds to NPR3 despite the fact that it lacks Cys.[20] An ANP homologous region in musclin resides between positions 8 and 22, with a Gly residue at position 7. A hybrid peptide of musclin and ANP, i.e., a Gly[7] analog of **1** (**7**), showed moderate binding affinity for NPR3

Table 1
Structures and binding affinities of natriuretic peptide receptor-3 blockers (R-AA[3]-AA[4]-AA[5]-AA[6]-AA[7]-AA[8]-AA[9]-AA[10]-AA[11]-AA[12]-AA[13]-AA[14]-AA[15]-AA[16]-C-ter.).

Compound	Structure																Binding affinity[a]	
	R	AA[3]	AA[4]	AA[5]	AA[6]	AA[7]	AA[8]	AA[9]	AA[10]	AA[11]	AA[12]	AA[13]	AA[14]	AA[15]	AA[16]	C-ter.	IC_{50} (nM)	
																	hNPR1	hNPR3
hANP																	0.094	0.058
C-ANP$_{4-23}$																	>1000	0.51
musclin (homology region)	–	Arg	Ser	Phe	Ser	Gly	Phe	Gly	Ser	Pro	Leu	Asp	Arg	Leu	Ser	–		
1	H-	–	–	–	–	Cys	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	120	1.5
2	Ac	–	–	–	–	Cys	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	35
3	H-	–	–	–	–	Ser	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	15
4	H-	–	–	–	–	Ala	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	13
5	H-	–	–	–	–	–	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	11
6	Ac	–	–	–	–	–	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	54
7	H-	–	–	–	–	Gly	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	13
8	H-	–	–	–	Ser	Gly	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	22
9	H-	–	–	Phe	Ser	Gly	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	1.2
10	H-	–	Ser	Phe	Ser	Gly	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	0.52
11	H-	Arg	Ser	Phe	Ser	Gly	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	0.30
12	H-	–	Arg	Ser	Ser	Gly	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	7.8
13	H-	–	–	D-Phe	Ser	Gly	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	0.12
14	Ac-	–	–	D-Phe	Ser	Gly	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	0.93
15	H-	–	–	D-Phe	Ser	D-Pro	Cha	Gly	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	0.13
16	H-	–	–	D-Phe	Ser	Gly	Cha	D-Ala	Gly	Arg	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	0.36
17	H-	–	–	D-Phe	Ser	Gly	Cha	Gly	Gly	Pro	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	0.12
18	Ac-	–	–	D-Phe	Ser	D-Pro	Cha	D-Ala	Gly	Pro	Ile	Asp	Arg	Ile	Gly	NH_2	>1000	0.19
19	Ac-	–	–	D-Phe	Ser	D-Pro	Cha	D-Ala	Gly	Pro	Ile	Asp	Arg	Ile	–	NH_2	>1000	0.054
20	Ac	–	–	D-Phe	Ser	D-Pro	Cha	D-Ala	Gly	Pro	Ile	Asp	Arg	Ile	–	$NHCH_3$	>1000	0.087
21	Ac	–	–	D-Phe	Ser	D-Pro	Cha	D-Ala	Gly	Pro	Ile	Asp	Arg(Me)	Ile	–	$NHCH_3$	>1000	0.14
22	Ac	–	–	D-Phe	Ser	D-Hyp	Cha	D-Ala	Gly	Hyp	Ile	Asp	Arg(Me)	Ile	–	$NHCH_3$	>1000	0.099
23	$HOCH_2CO$	–	–	D-Phe	Ser	D-Hyp	Cha	D-Ser	Gly	Hyp	Ile	Asp	Arg(Me)	Ile	–	$NHCH_3$	>1000	0.079

[a] IC_{50} values of peptides represent the concentrations required to displace the binding of radiolabeled ligand by 50%.