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Design and evaluation of novel natriuretic peptide derivatives with improved pharmacokinetic and pharmacodynamic properties

Naomi Morozumi[a,*], Seiji Sato[a], Sayaka Yoshida[a], Yuriko Harada[a], Mayumi Furuya[a], Yoshiharu Minamitake[a], Kenji Kangawa[b]

[a] Asubio Pharma Co, Ltd. 6-4-3 Minatojima-Minamimachi, Chuo-ku, Kobe, 650-0047, Japan
[b] National Cerebral and Cardiovascular Center Research Institute, 5-7-1, Fujishirodai, Suita, Osaka, 565-8565, Japan

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ABSTRACT

C-type natriuretic peptide (CNP) and its receptor, natriuretic peptide receptor B (NPR-B), are potent positive regulators of endochondral bone growth, making the CNP pathway one of the most promising therapeutic targets for the treatment of growth failure. However, the administration of exogenous CNP is not fully effective, due to its rapid clearance in vivo. Modification of CNP to potentially druggable derivatives may result in increased resistance to proteolytic degradation, longer plasma half-life ($T_{1/2}$), and better distribution to target tissues. In the present study, we designed and evaluated CNP/ghrelin chimeric peptides as novel CNP derivatives. We have previously reported that the ghrelin C-terminus increases peptide metabolic stability. Therefore, we combined the 17-membered, internal disulfide ring portion of CNP with the C-terminal portion of ghrelin. The resultant peptide displayed improved biokinetics compared to CNP, with increased metabolic stability and longer plasma $T_{1/2}$. Repeated subcutaneous administration of the chimeric peptide to mice resulted in a significant acceleration in longitudinal growth, whereas CNP(1–22) did not. These results suggest that the ghrelin C-terminus improves the stability of CNP, and the chimeric peptide may be useful as a novel therapeutic agent for growth failure and short stature.

1. Introduction

C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family, along with atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). ANP and BNP are circulating hormones mainly produced in the heart, and regulate body fluid volume and blood pressure [13]. On the other hand, the expression of CNP is regulated in a tissue-specific manner, and is detected at highest levels in the central nervous system (CNS), female reproductive tissues, and bone [6,16]. The peptide concentration in circulating plasma is very low, therefore it has been suggested that CNP is an autocrine/paracrine regulator of the CNS and/or peripheral tissues [14,16]. It is well recognized that CNP and its receptor, natriuretic peptide receptor B (NPR-B) are potent stimulators of endochondral ossification [20,21]. Previous reports using genetically engineered animal models have revealed the physiological role of the local CNP/NPR-B system in growth plates. CNP or NPR-B knockout (KO) mice display dwarfism due to impaired endochondral ossification, whereas overexpression of CNP causes skeletal overgrowth in mice [2,14,19–21]. Based on these results, CNP has been extensively investigated as a therapeutic candidate for the treatment of growth failure and short stature disorders such as achondroplasia [20,21]. However, therapeutic targeting of the CNP/NPR-B system has not yet achieved clinical success, due to the short plasma half-life ($T_{1/2}$) of CNP(1–22), the endogenous form of CNP found in vivo [7,18]. CNP(1–22) is rapidly hydrolyzed by neutral endopeptidase (NEP) present in the plasma and on endothelial cell surfaces [8].

Ghrelin is a bioactive peptide that is predominantly secreted from the stomach, which regulates growth hormone (GH) secretion, appetite, digestive function, and bone metabolism through systemic circulation [3,9,10,22]. We have demonstrated in previous studies that the C-terminal portion of ghrelin has an essential role in GH secretion activity [11], and that chimeric peptides composed of the N-terminal portion of motilin and the C-terminal portion of ghrelin were more stable than the native form of motilin in vivo [12]. Hence, we hypothesized that the

Abbreviation: ANP, atrial natriuretic peptide; AUC, The area under the plasma concentration-time curve; BNP, brain natriuretic peptide; cGMP, Cyclic guanosine monophosphate; CHO, Chinese hamster ovary; CNP, C-type natriuretic peptide; CNS, the central nervous system; GH, growth hormone; GPCR, G-protein-coupled receptors; HPLC, high-performance liquid chromatography; iv, intravenous; KO, knockout; M.W., molecular weight; NEP, neutral endopeptidase; NPR-B, natriuretic peptide receptor B; PBS, Phosphate buffered saline; PD, pharmacodynamics; PK, pharmacokinetics; sc, subcutaneous; RIA, radio-immuno assay; SD, standard deviation; $T_{1/2}$, half-life

* Corresponding author.
E-mail address: morozumi.naomi.dg@asubio.co.jp (N. Morozumi).

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functional characteristics of the ghrelin C-terminus may make it a useful component of a novel CNP derivative with improved metabolic stability. In this study, we have prepared several CNP/ghrelin chimeric peptides and evaluated their pharmacokinetic (PK) and pharmacodynamic (PD) profiles.

In the following sections, we present the structure-activity relationships of CNP/ghrelin chimeric peptides, and the effects of daily subcutaneous (sc) injections of a chimeric peptide on longitudinal growth in normal mice.

2. Materials and methods

2.1. Peptides

CNP(1–22) was produced from recombinant DNA in Escherichia coli, and CNP/ghrelin chimeric peptides were chemically synthesized using a solid phase method. They were purified by high-performance liquid chromatography (HPLC), and the purity of each peptide was over 95%. Peptides were verified by amino acid composition analysis, amino acid sequence analysis, and/or electrospray ionization mass spectrometry.

2.2. Animals

We used 7-week-old male Sprague-Dawley rats and 3-week-old female ICR mice (Charles River Laboratories, Japan) in this study. The animals were housed in a humidity- and temperature-controlled environment with an automatic 12-h light/dark cycle. They were fed a standard, pelleted lab chow diet (CRF-1, Oriental Yeast Co., Ltd., Japan) and tap water ad libitum. All experiments were performed with the approval of the Ethics Committee of Asubio Pharma Co., Ltd.

2.3. In vitro cyclic guanosine monophosphate (cGMP) production assay

To evaluate the NPR-B agonist activities of the test compounds, we used Chinese hamster ovary (CHO) cells stably expressing human NPR-B. Briefly, the cells were placed on flat-bottomed 96-well plates at a density of 1×10^4 cells/well for 1 day. The test compounds were dissolved in distilled water or 0.1 N acetic acid, and diluted with phosphate buffered saline (PBS). Prior to measurement, the cells were pretreated with 80 µl/well of 0.75 mM 3-isobutyl-1-methylxanthine in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 10 mM glucose for 10 min. Then, 40 µl/well of the test compound solution or PBS (as a negative control) was added and incubated for 15 min at 37 °C, before 40 µl lysis buffer was added with mixing. The solution in each well was collected and frozen at −20 °C or below.

The cGMP concentration in each sample was determined by competitive ELISA using the CatchPoint cyclic-GMP Fluorescent Assay Kit and FlexStation™ (Molecular Devices Corporation, U. S. A.), following the manufacturer's instructions.

2.4. NEP resistance assay

Test solutions of CNP(1–22) and CNP(6–22)Ghrelin(12–28) (0.5 µg/ml) were incubated in the presence of purified recombinant human NEP (R & D Systems, U. S. A.) in 20 mM 2-(N-morpholino)ethanesulfonic acid buffer (pH 6.5) at 37 °C for 60 min. After the incubation, 100 µl of the test solution was boiled for 5 min, and 100 µl of distilled water was added. All samples were analyzed using a water-acetonitrile gradient on an HPLC (LC-10A, Shimadzu Co., Japan). Results were reported as the percentage of intact peptide remaining compared with time zero. Each peptide was evaluated in duplicate assays.

2.5. PK studies in rats

Rats were weighed and anesthetized by intraperitoneal injection of sodium pentobarbital (30–50 mg/kg, Sumitomo Dainippon Pharma Co., Ltd., Japan). A catheter was placed in the femoral artery of each rat to collect blood samples. The test compounds were weighed and dissolved in distilled water, and diluted with 5% mannitol solution at use. The rats received 10 nmol/kg of each compound by a single intravenous (iv) bolus injection in the tail vein.

In the combination study with a NEP inhibitor, the rats received either 20 µg/kg (9.1 or 5.3 nmol/kg) of CNP(1–22) or CNP(6–22)ghrelin(12–28) by a single iv bolus injection combined with an intrafemoral vein infusion of either 5% mannitol or DL-Thiorphan, a NEP inhibitor, (30 µg min^{-1} $body^{-1}$, Sigma-Aldrich Co. LLC., U. S. A.) using the constant infusion syringe pump (CFV2100, Nihon Kohden Corporation, Japan).

All blood samples were treated with 1/100 vol of 10% ethylenediaminetetraacetic acid (Wako Pure Chemical Industries, Ltd., Japan) and 5000 U/ml aprotinin (Bayer Yakuhin, Ltd, Japan) to protect peptides from proteases. After centrifugation at 12 000 rpm for 5 min at 4 °C, the plasma samples were collected. CNP immunoreactivity in plasma was determined by radioimmuno assay (RIA) using antiserum recognizing the 17-membered, internal disulfide ring of CNP and [^{125}I]-labeled [Tyr^0]-CNP(1–22) as a tracer. Each peptide was used as the standard for itself in the assay. The cGMP concentration in plasma was determined by RIA using the Yamasa Cyclic GMP Assay Kit (YAMASA Corporation, Japan).

2.6. Pharmacological effects in mice

Three-week-old ICR female mice (n = 10 or 8/group) received sc injections of CNP(1–22), CNP(6–22)ghrelin(12–28), or vehicle [30 mM acetic acid pH 4.0 containing 10% (w/v) sucrose and 1% (w/v) benzyl alcohol] twice or thrice a day for 29 or 30 days. Growth was monitored throughout the treatment period, and we measured body weight, body (naso-anal) length, and tail length. Long bones of limbs were collected after euthanasia at the final day and their lengths were measured with digital calipers.

2.7. Data analysis

The $T_{1/2}$ of immunoreactivity in plasma was calculated individually by the least-squares method. Area under the plasma concentration-time curve (AUC) was calculated by the area of the trapezoids defined by pairs of points and the points along the x-axis. All data are expressed as the mean or the mean ± standard deviation (SD). Statistical differences in body and bone lengths between mice in the control and treatment groups were analyzed using Tukey-Kramer's test with JMP® 10 software (SAS Institute Japan Ltd.). A P value of < 0.05 was considered to indicate statistically significant differences.

3. Results

3.1. PK/PD profiles of CNP/ghrelin chimeric peptides in rats

We designed several CNP/ghrelin chimeric peptides to evaluate the relationship between the attachment of the C-terminal of ghrelin and the PK/PD profiles of the chimeric peptides. Their amino acids sequences and the molecular weights (M.W.) are shown in Table 1.

Fig. 1A shows the cGMP production activity of chimeric peptides in CHO cells stably expressing human NPR-B. All peptides activated human NPR-B and enhanced the production of cGMP.

When the C-terminal of ghrelin was attached to the monolateral side of the 17-membered internal disulfide ring of CNP [CNP(6–22)], the activity of the NPR-B agonist decreased compared to that of CNP(1–22). Among these, the CNP(6–22)ghrelin(12–28)amide maintained agonist activity comparable to that of CNP(1–22), suggesting that C-terminal amidation might be effective for enhancing activity. CNP(6–22)ghrelin (12–28) exhibited NPR-B activity within one-tenth of that observed for