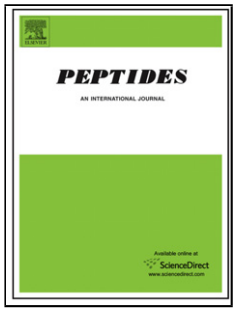

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Design and evaluation of novel natriuretic peptide derivatives with improved pharmacokinetic and pharmacodynamic properties

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Highlights

- Verification and evaluation of properties of the C-terminal portion of ghrelin
- Approaches to improve stability, bioavailability and bioactivity of CNP
- CNP/ghrelin chimeric peptide as a new therapeutic candidate for growth failure

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.

Abbreviations used:

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

Keywords:

ghrelin, C-type natriuretic peptide, chimeric peptide, endochondral ossification, pharmacokinetics, metabolic stability

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 [3,10,22]. 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 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-hour 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 pre-treated 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 minutes. Then, 40 µl/well of the test compound solution or PBS (as a negative control) was added and incubated for 15 minutes 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 minutes. After the incubation, 100 µl of the test solution was boiled for 5 minutes, 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 intra-femoral vein infusion of either 5% mannitol or DL-Thiorphan, a NEP inhibitor, (30 $\mu g \cdot min^{-1} \cdot body^{-1}$, 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 volume 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 minutes 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 CNP(1-22); however, the activity of ghrelin(12-28)CNP(6-22) was about ten-fold less potent than that of CNP(6-22)ghrelin(12-28). Ghrelin(28-12)CNP(6-22), which consists of the reversed sequence ghrelin(28-12) fused to the N-terminal side of CNP(6-22), showed higher potency than that having the forward sequence [ghrelin(12-28)CNP(6-22)]. A linker consisting of the middle region of ghrelin might be necessary for retaining of its agonist activity.

Furthermore, ghrelin(28-12)CNP(6-22)ghrelin(12-28) exhibited lower potency than that of ghrelin(28-12)CNP(6-22) and CNP(6-22)ghrelin(12-28). Additionally, the agonist activity of ghrelin(12-28)CNP(6-22)ghrelin(12-28) was also the lowest. The bilateral fusion peptides might be too large to access to the binding pocket of the receptor.

No agonist or antagonist action on the ghrelin/GHS-R pathway was detected for all chimeric peptides produced (data not shown).

In the PK study, after an iv injection of each peptide into rats at 10 nmol/kg, plasma CNP immunoreactivity-time curves were generated as shown in Fig. 1B, and the $AUC_{0\text{-}60min}$ and $T_{1/2}$ of the test compounds are shown in Table 2.

With all chimeric peptide doses, the plasma CNP immunoreactivity was higher than that of the native CNP(1-22). The $T_{1/2}$ of the chimeric peptides varied in the range of 14.50 and 18.40 min, over three-fold longer than the $T_{1/2}$ of CNP(1-22), which was 4.34 min. The $AUC_{0\text{-}60min}$ values were 7.3-23.6 times larger than that of CNP(1-22).

There was little difference in the PK profiles of CNP(6-22)ghrelin(12-28), ghrelin(12-28)CNP(6-22), and ghrelin(28-12)CNP(6-22). On the other hand, the AUC of CNP(6-22)ghrelin(12-28)amide was three-tenths smaller than that of the non-amidated CNP(6-22)ghrelin(12-28). The AUC of ghrelin(28-12)CNP(6-22)ghrelin(12-28) was two-fold larger than that of ghrelin(12-28)CNP(6-22)ghrelin(12-28).

Although the AUC values had about 3-fold differences among the chimeric peptides, there were no notable changes in the $T_{1/2}$. We hypothesized that these peptide sequences might affect their distribution volume.

In particular, CNP(6-22)ghrelin(12-28) demonstrated a superior PK/PD profile, and the plasma cGMP levels after a dose of CNP(6-22)ghrelin(12-28) were markedly higher than that after a dose of native CNP(1-22) at all time points (Fig. 1C). Therefore, we selected CNP(6-22)ghrelin(12-28) as the leading compound for clinical development for further evaluations.

.3.2 NEP resistance of CNP/ghrelin chimeric peptides

Based on the results of the PK/PD analysis, CNP(6-22)ghrelin(12-28) was selected for NEP resistance assay. After incubation with purified human NEP, the residual amounts of intact peptides were 16.7 and 83.8% in CNP(1-22) and CNP(6-22)ghrelin(12-28), respectively. These results indicated that CNP(6-22)ghrelin(12-28) was resistant to human NEP.

Plasma CNP immunoreactivity concentration-time curves after a single iv injection of CNP(1-22) or CNP(6-22)ghrelin(12-28) are shown in Fig. 2. Coadministration of the NEP inhibitor significantly increased the plasma level in rats that received CNP(1-22), as the AUC and $T_{1/2}$ of CNP(1-22) were 3.7-fold higher and 5-fold longer, respectively, than without the inhibitor (Table 3). Conversely, a significant change was not observed for CNP(6-22)ghrelin(12-28) in the presence of the NEP inhibitor. The $T_{1/2}$ of CNP immunoreactivity after the injection of CNP(6-22)ghrelin(12-28) without or in the presence of the inhibitor was 14.9 ± 3.1 or 12.7 ± 1.2 min, longer than with coadministration of CNP(1-22) and the NEP inhibitor. These results indicate that the ghrelin C-terminus stabilized and prolonged the plasma $T_{1/2}$ of CNP(6-22)ghrelin(12-28).

3.3 The CNP/ghrelin chimeric peptide promotes bone growth in mice

When 3-week-old female ICR mice (n=10/group) were given sc injections of CNP(1-22) (0.25 mg/114 nmol/kg, twice daily) or CNP(6-22)ghrelin(12-28) (0.25 mg/67 nmol/kg, twice daily) for 29 days, the body and tail lengths of mice in the CNP(6-22)ghrelin(12-28) group were significantly extended (Table 4), while the CNP(1-22) group displayed no differences from the control group. There were no differences in body weight among the groups. No antibodies against CNP(1-22) or CNP(6-22)ghrelin(12-28) were detected in the mice after the final dosing (data not shown).

Next, CNP(6-22)ghrelin(12-28) was repeatedly administered sc to 3-week-old female ICR mice (n=8/group) at doses of 0.1 or 0.5 mg/kg (27 or 133 nmol/kg, thrice daily) for 30 days. CNP(6-22)ghrelin(12-28) significantly stimulated appendicular and axial skeletal growth in a dose-dependent manner (Fig. 3). At doses of 0.5 mg/kg, CNP(6-22)ghrelin(12-28) had powerful and proportional effects on the lengths of the humerus, ulna, femur, and tibia bones of mice, all of which were significantly longer than those of the vehicle control group.

4. Discussion

CNP is a potent stimulator of endochondral bone growth [20], but administration of exogenous CNP is not fully effective due to its rapid clearance in vivo [7,8]. In this study, we designed novel natriuretic peptide derivatives taking advantage of the stabilizing effects of the ghrelin C-terminus, and discovered a novel CNP/ghrelin chimeric peptide with increased stability, prolonged $T_{1/2}$, and potent effects on skeletal growth in vivo.

Previously, we demonstrated that the ghrelin C-terminus plays an important role in maintaining its stability [11], and in addition, motilin/ghrelin chimeric peptides increase the plasma stability of motilin without affecting its bioactivity [12]. Both ghrelin and motilin are mainly secreted from the gastrointestinal tract and act on structurally homologous G protein-coupled receptors (GPCRs) [17]. The amino acids sequences of ghrelin and motilin are similar, and these peptides are considered members of the motilin-ghrelin peptide family [1]. Therefore, it might be relatively easy to replace a portion of one structure with each other.

CNP is a ligand for the NPR-B receptor, which is distinct from GPCRs [15]. The structure and function of CNP are also completely different from those of ghrelin. The 17-membered, internal disulfide ring of CNP (CNP(6-22)) alone is sufficient for the stimulation of cGMP accumulation via NPR-B [5]. Therefore, we synthesized several CNP analogs with CNP(6-22) and the ghrelin C-terminus, and measured their biological actions.

CNP/ghrelin chimeric peptides demonstrated superior PK profiles compared with native CNP(1-22), regardless of the conformation or bonding system used. For example, when the amino acid sequence was reversed, and even when the C -terminal end was amidated for stabilization, the extended plasma $T_{1/2}$ was maintained. Although NPR-B agonist activity was lowered in some derivatives, we did observe chimeric peptides with agonist activity (Fig. 1, Table 1). CNP is known to be metabolized by NEP, but CNP derivatives containing the C-terminal portion of ghrelin are resistant to NEP (Fig. 2, Tables 3).

The C-terminal tail structures of ANP and BNP affect their biological activity and receptor selectivity [4]. However, the ghrelin C-terminus had little effect on the receptor specificity of CNP. One of the most simply structured chimeric peptides, CNP(6-22)ghrelin(12-28), had full agonist activity for NPR-B and an extended plasma $T_{1/2}$ in vivo. It significantly stimulated longitudinal growth in mice by sc injection in a dose-dependent manner.

Our present study reveals that addition of the ghrelin C-terminus to CNP improves its PK/PD characteristics in vivo. The C-terminal portion could be applied to either the N- or C-terminal of CNP in either direction, suggesting that this sequence might have wide applications, improving the metabolic stability of various peptide hormones while maintaining their original biological activity. These results also indicate that CNP/ghrelin chimeric peptides maintained an important physiological characteristic of ghrelin, acting as circulating peptide hormones without loss of bioactivity. While therapeutic treatment of short stature is ineffective with native CNP, the addition of the ghrelin C-terminus may provide CNP with enhanced pharmacological action.

5. Conclusions

In this study, we have demonstrated that adding the C-terminal portion of ghrelin to CNP produced CNP/ghrelin chimeric peptides more stable than native CNP, with improved bioactivity in vivo. CNP/ghrelin chimeric peptides may be useful as therapeutic agents for bone-related diseases such as short stature and achondroplasia.

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Figure and Table legends

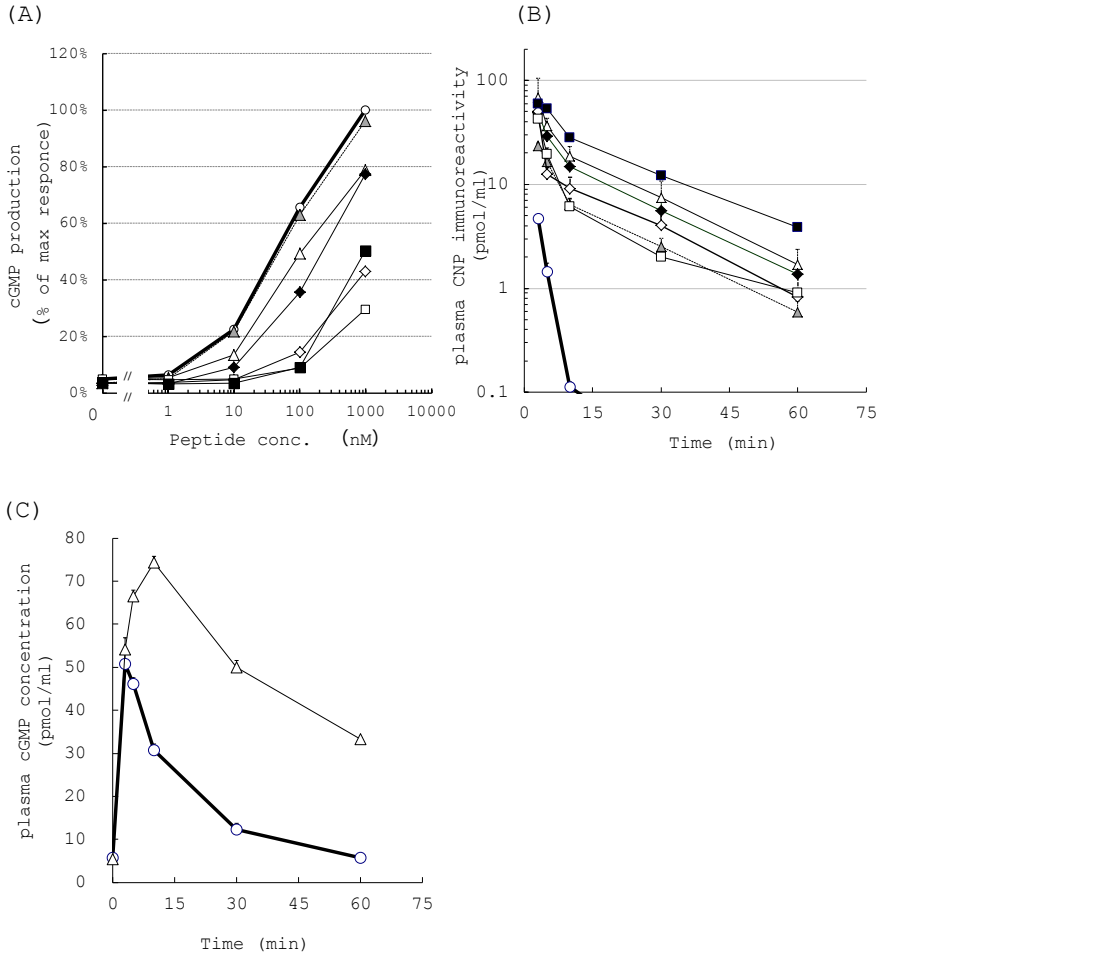

Fig.1

Human NPR-B agonist activity and PK profile of CNP(1-22) and CNP/ghrelin chimeric peptides. (A) cGMP production activity in CHO cells stably expressing human NPR-B. Each value represents the mean of duplicate determinations. (B) Plasma CNP immunoreactivity concentration-time curves after the single iv administration of CNP(1-22) or CNP/ghrelin chimeric peptides(10 nmol/kg) to male rats. Each value represents the mean + SD of 3 rats. Plasma CNP immunoreactivity was determined by RIA. (C) Plasma cGMP concentration-time curves after the single iv administration of CNP(1-22) or CNP(6-22)ghrelin(12-28) (10 nmol/kg) to male rats. Each value represents the mean +SD of 3 rats.

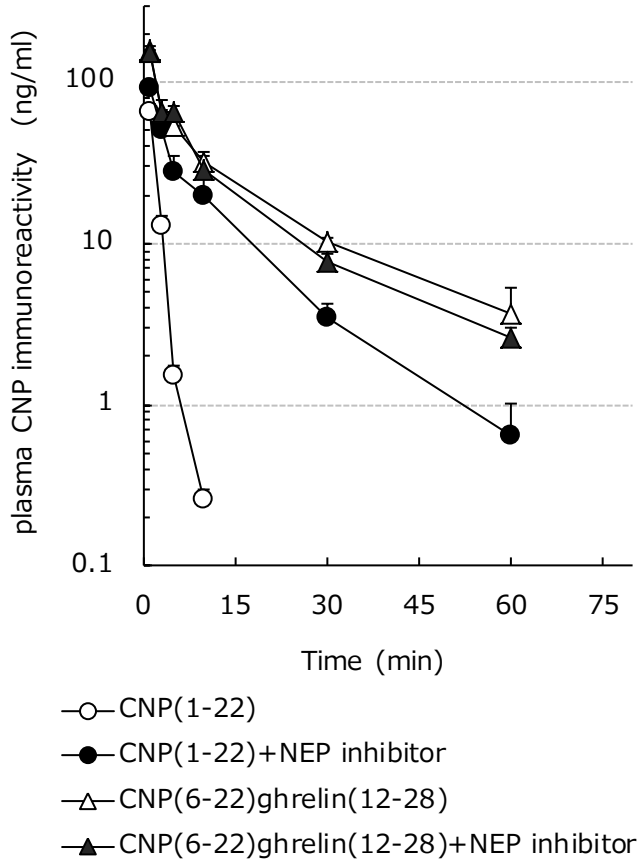

Fig.2

Effect of NEP inhibitor co-administration on CNP immunoreactivity concentration-time curves in plasma after the single iv injection of CNP(1-22) or CNP(6-22)ghrelin(12-28) (0.02 mg/kg) combined with iv infusion of either 5% mannitol or DL-Thiorphan ($30\ \mu g \cdot min^{-1} \cdot body^{-1}$) to male rats. Each value represents the mean +SD of 3 rats.

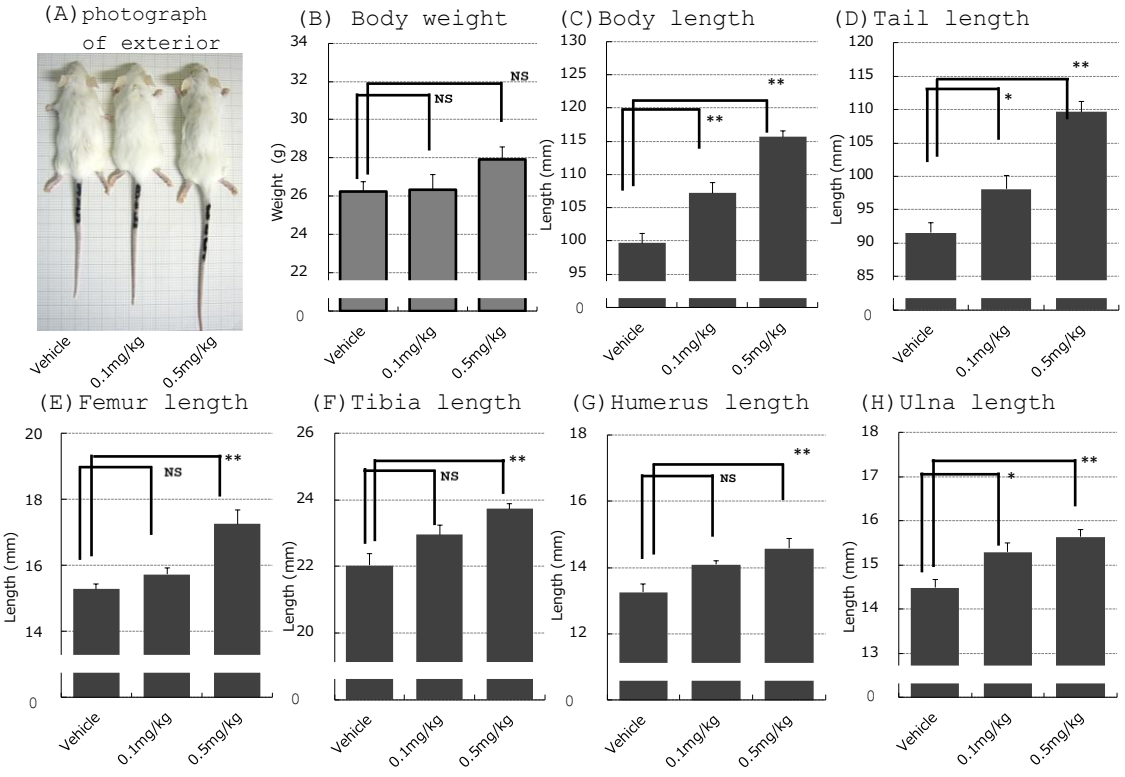

Fig.3

Body weight, body length and tail length of female mice after the repeated sc administration of CNP(6-22)ghrlein(12-28) at doses of 0.1 or 0.5 mg/kg thrice daily for 30 days. Each value represents the mean + SD of 8 mice. NS: not significant($P>0.05$), *: significant($p<0.05$) and **: significant($p<0.01$) with Tukey-Kramer's HSD test

Table 1

Amino acids sequences and molecular weight of CNP(1-22) and various CNP/ghrelin chimeric peptides.

Peptides (Molecular Weight)	Amino acid sequence[a,b]
CNP(1-22) (2197.6)	GLSKG**C**FGLKLDRIGSMSGLG**C**
CNP(6-22)ghrelin(12-28) (3755.5)	**C**FGLKLDRIGSMSGLG**C**VQQRKESKKPPAKLQPR
ghrelin(12-28)CNP(6-22) (3755.5)	VQQRKESKKPPAKLQPR**C**FGLKLDRIGSMSGLG**C**
ghrelin(28-12)CNP(6-22) (3755.5)	RPQLKAPPKKSEKRQQV**C**FGLKLDRIGSMSGLG**C**
CNP(6-22)ghrelin(12-28)amide (3754.5)	**C**FGLKLDRIGSMSGLG**C**VQQRKESKKPPAKLQPR-amide
ghrelin(12-28)CNP(6-22)ghrelin(12-28) (5755.9)	VQQRKESKKPPAKLQPR**C**FGLKLDRIGSMSGLG**C**VQQRKESKKPPAKLQPR
ghrelin(28-12)CNP(6-22)ghrelin(12-28) (5755.9)	RPQLKAPPKKSEKRQQV**C**FGLKLDRIGSMSGLG**C**VQQRKESKKPPAKLQPR

a One-letter amino acid notation is used

b **C** : disulfide bond site

Table 2

PK parameters of CNP(1-22) and various CNP/ghrelin chimeric peptides in rats. Each value of AUC and $T_{1/2}$ represents the mean +/- SD of 3 rats.

Peptides	$AUC_{0-60min}$ (pmole·min/ml)		$T_{1/2}$ (min)	
CNP(1-22)	45	± 2	4.34	± 0.20
CNP(6-22)ghrelin(12-28)	1009	± 52	15.03	± 4.31
ghrelin(12-28)CNP(6-22)	705	± 84	17.75	± 3.99
ghrelin(28-12)CNP(6-22)	734	±100	14.86	± 2.64
CNP(6-22)ghrelin(12-28)amide	330	± 37	14.50	± 0.85
ghrelin(12-28)CNP(6-22)ghrelin(12-28)	489	± 52	18.40	± 2.05
ghrelin(28-12)CNP(6-22)ghrelin(12-28)	1060	±160	17.38	± 1.00

AUC was calculated by the area of the trapezoids defined.

$T_{1/2}$ was calculated individually by the least-squares method.

Table 3

Pharmacokinetic parameters of CNP(1-22) and CNP(6-22)ghrelin(12-28) in rats. The native CNP(1-22) or CNP(6-22)ghrelin(12-28) was intravenously administered at a dose of 0.02 mg/kg. Either 5% mannitol or DL-Thiorphan (30 μg·min^{-1}·body^{-1}) was infused into the femoral vein. Each value represents the mean +/- SD of 3 rats.

Peptides	Dose (mg/kg)	NEP inhibitor	$AUC_{0\rightarrow\infty}$ (ng・min/ml)	$T_{1/2}$ (min)
CNP(1-22)	0.02	-	202± 24	1.96±0.05
		+	758± 88	9.94±1.40
CNP(6-22)ghrelin(12-28)	0.02	-	1470± 3	14.9 ±3.1
		+	1342±168	12.7 ±1.2

AUC was calculated by the area of the trapezoids defined.

$T_{1/2}$ was calculated individually by the least-squares method.

Table 4

Effect of CNP(1-22) and CNP(6-22) ghrelin(12-28) on the body weight and the longitudinal growth of ICR mice. The native CNP(1-22) or CNP(6-22)ghrelin(12-28) was repeatedly administered subcutaneously at a dose of 0.25 mg/kg twice daily for 29 days. Each value represents the mean +/- SD of 10 mice.

Test compounds (Peptides)	Body weight (g)	Body length (mm)	Tail length (mm)
- (Vehicle control)	28.0 ±2.1	102.9 ±2.6	90.7 ±4.0
CNP(1-22)	27.2 ±1.4^{NS}	103.0 ±2.1^{NS}	90.7 ±2.8^{NS}
CNP(6-22)ghrelin(12-28)	27.5 ±1.9^{NS}	109.5 ±2.7**	96.8 ±3.5**

NS: not significant(P>0.05), **: Significant(p<0.01) with Tukey-Kramer's HSD test