

Ipamorelin, the first selective growth hormone secretagogue

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Abstract

The development and pharmacology of a new potent growth hormone (GH) secretagogue, ipamorelin, is described. Ipamorelin is a pentapeptide (Aib-His-D-2-Nal-D-Phe-Lys-NH₂), which displays high GH releasing potency and efficacy *in vitro* and *in vivo*. As an outcome of a major chemistry programme, ipamorelin was identified within a series of compounds lacking the central dipeptide Ala-Trp of growth hormone-releasing peptide (GHRP)-1.

In vitro, ipamorelin released GH from primary rat pituitary cells with a potency and efficacy similar to GHRP-6 (EC₅₀ = 1.3±0.4 nmol/l and E_{max} = 85±5% vs 2.2±0.3 nmol/l and 100%). A pharmacological profiling using GHRP and growth hormone-releasing hormone (GHRH) antagonists clearly demonstrated that ipamorelin, like GHRP-6, stimulates GH release via a GHRP-like receptor.

In pentobarbital anaesthetised rats, ipamorelin released GH with a potency and efficacy comparable to GHRP-6 (ED₅₀ = 80±42 nmol/kg and E_{max} = 1545±250 ng GH/ml vs 115±36 nmol/kg and 1167±120 ng GH/ml).

In conscious swine, ipamorelin released GH with an ED₅₀ = 2.3±0.03 nmol/kg and an E_{max} = 65±0.2 ng GH/ml plasma. Again, this was very similar to GHRP-6 (ED₅₀ = 3.9±1.4 nmol/kg and E_{max} = 74±7 ng GH/ml plasma). GHRP-2 displayed higher potency but lower efficacy (ED₅₀ = 0.6 nmol/kg and E_{max} = 56±6 ng GH/ml plasma).

The specificity for GH release was studied in swine. None of the GH secretagogues tested affected FSH, LH, PRL or TSH plasma levels. Administration of both GHRP-6 and GHRP-2 resulted in increased plasma levels of ACTH and cortisol. Very surprisingly, ipamorelin did not release ACTH or cortisol in levels significantly different from those observed following GHRH stimulation. This lack of effect on ACTH and cortisol plasma levels was evident even at doses more than 200-fold higher than the ED₅₀ for GH release.

In conclusion, ipamorelin is the first GHRP-receptor agonist with a selectivity for GH release similar to that displayed by GHRH. The specificity of ipamorelin makes this compound a very interesting candidate for future clinical development.

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Introduction

Growth hormone (GH) has been used for treating various types of growth disorders for a number of years. Following the availability of recombinant GH in 1985, the unlimited supply has made it possible to investigate the more general metabolic effects of GH. Currently, a number of data suggests benefits of using GH in the frail elderly, osteoporosis, in catabolic states and wasting conditions, e.g. following surgery (1, 2). Convincing effects following GH treatment in obesity have also been reported (3).

Somatotrophs in the anterior pituitary gland release GH in a pulsatile fashion under the regulation of two hypothalamic peptides: GH-releasing hormone (GHRH) and somatostatin. GHRH stimulates GH synthesis and secretion, while somatostatin inhibits GH release without affecting GH synthesis (4, 5). In addition, a third

system, the GH-releasing peptide (GHRP) system also seems to be involved in the regulation of GH release (6, 7). Over the past years a growing interest in GHRPs has evolved. It has been suggested that this novel class of GH secretagogues, including GHRP-6 (6) and MK-677 (8), offer an advantage compared with exogenously administered GH, by increasing plasma GH levels in a more physiologically relevant, pulsatile fashion (9).

A number of GH secretagogues have proven efficacious in elevating plasma GH in animals, e.g. in rats (10), dogs (11) and swine (12). More recently, GHRP-2 (13), GHRP-6 (14), hexarelin (15), and MK-677 (16) have also been shown to elevate plasma GH in humans. All of these GH secretagogues potently release GH and, in addition, prolactin (PRL) and cortisol, the latter probably indirectly by stimulating adrenocorticotrophin (ACTH) (17, 18).

In the present study we describe the development of a novel series of GHRP receptor-active GH secretagogues. A prototype of this series, ipamorelin, releases GH with high potency and efficacy without significant effects on plasma ACTH or cortisol levels, compared with the main endogenous GH releasing compound, GHRH. Consequently, ipamorelin is the most selective GHRP receptor-active GH secretagogue described.

Materials and methods

Chemistry

The secretagogues shown in Table 1 were synthesised stepwise on a solid support using the Boc peptide synthesis strategy on an Applied Biosystems Model 430A peptide synthesizer or by using the Fmoc strategy on an Applied Biosystems Model 431A peptide synthesizer. The standard protocols supplied by the manufacturer were used. In all cases the crude peptides were purified using semi-preparative RP-HPLC to a purity of >95% (based on HPLC with detection at 214 nm). The compounds were all characterised by analytical HPLC and by plasma desorption mass spectrometry. The molecular mass found was in agreement with the expected value in all cases.

In particular, ipamorelin was prepared from a total of 4.53 g of the peptide resin Boc-Aib-His (Trt)-D-2Nal-D-Phe-Lys (Boc)-NH-resin according to the Fmoc strategy on an Applied Biosystems 431A peptide synthesizer in two identical runs (to obtain sufficient material) in 1 mmol scale using the FastMoc UV protocols supplied by the manufacturer, that employ HBTU mediated couplings in NMP and UV monitoring of the deprotection of the Fmoc protection group.

The starting resin used for the synthesis was 2×1.75 g (4-((2',4'-dimethoxyphenyl)-(Fmoc-amino)-methyl)-phenoxy resin (Rink resin) (Novabiochem, Bad Soden, Germany, cat. no. 01-64-0013) with a substitution capacity of 0.55 mmol/g. The protected amino acid derivatives used were Fmoc-Lys(Boc)-OH, Fmoc-D-Phe-OH, Boc-D-2Nal-OH, Boc-His(Trt)-OH and

Boc-Aib-OH (Boc-Amino isobutyric acid). The peptide was cleaved from the 4.53 g peptide resin by treatment with 54 ml TFA/phenol/ethanedithiol/thioanisole/water 40:3:1:2:2 for 3 h at room temperature.

Purification was carried out by semi-preparative RP-HPLC and the final product was characterised by amino acid analysis, analytical RP-HPLC and by plasma desorption mass spectrometry. Amino acid analysis and mass spectrometry were in agreement with the expected structure and within the experimental error of the method (mass spectrometry ±0.9 amu, amino acid analysis ±10%). In addition ¹H-NMR analysis was performed and the spectra assigned. The NMR spectra fully supported the proposed structure.

Ipamorelin is a white amorphous powder isolated as a trifluoroacetate. Its solubility in distilled water is >200 mg/ml. The pKas of the compound are 10.3, 7.8, and 6.2.

Rat pituitary cell assay

Sprague-Dawley male albino rats (250±25 g) were purchased from Møllegaard, Lille Skensved, Denmark. The rats were housed in group cages (four to eight animals/cage) and placed in rooms with a 12-h light cycle. The room temperature varied from 19–24 °C and the humidity from 30–60%. All media were obtained from Gibco (Paisley, UK), trypsin from Worthington (NJ, USA), BSA, DNase, tri-iodothyronine (T₃) and dexamethasone from Sigma (St Louis, MO, USA).

Cultures of pituitary cells were prepared according to Heiman *et al.* (19). Briefly, the rats were decapitated and the pituitaries dissected. The neurointermediate lobes were removed and the remaining tissue was immediately placed in ice-cold isolation buffer (Gey's medium (Gibco) supplemented with 0.25% D-glucose, 2% non-essential amino acids and 1% BSA, pH 7.3). The tissue was cut into small pieces and transferred to isolation buffer supplemented with 3.8 mg/ml trypsin and 330 µg/ml DNase. This mixture was incubated at 70 rotations/min for 35 min at 37 °C in a 95%:5% atmosphere of O₂:CO₂. The tissue was then washed three

Table 1 Structure–activity relations of some novel pentapeptides on GH release from rat pituitary cells *in vitro* – a comparison with GHRP-1, GHRP-2, GHRP-6 and GHRH. The results are shown as means ± s.e.m. (n = 3–6 separate experiments).

Secretagogue	Structure	EC ₅₀ (nmol/l)	E _{max} (%)
GHRP-1	Ala-His-D-2-Nal-Ala-Trp-D-Phe-Lys-NH ₂	1.1 ± 0.3	80 ± 5
NNC 26-0039	Ala-His-D-2-Nal-.....-D-Phe-Lys-NH ₂	6.5 ± 1.7	105 ± 13
NNC 26-0133	D-Ala-His-D-2-Nal-.....-D-Phe-Lys-NH ₂	15 ± 8	60 ± 8
Ipamorelin	Aib-His-D-2-Nal-.....-D-Phe-Lys-NH ₂	1.3 ± 0.4	85 ± 5
GHRP-2	Ala-.....-D-Trp-Ala-Trp-D-Phe-Lys-NH ₂	1.8 ± 0.5	85 ± 8
GHRP-6	His-D-Trp-Ala-Trp-D-Phe-Lys-NH ₂	2.2 ± 0.3	100
GHRH		0.26 ± 0.07	126 ± 7

E_{max} value for GHRP-6 = 231 ± 51 ng/ml (n = 6).

times in the above buffer. Using a standard Pasteur pipette, the tissue was then aspirated into single cells. After dispersion, cells were filtered through a nylon filter (160 μm) to remove undigested tissue. The cell suspension was washed 3 times with isolation buffer supplemented with trypsin inhibitor (0.75 mg/ml) and finally resuspended in culture medium; Dulbecco's modified Eagle's medium supplemented with 25 mmol/l HEPES, 4 mmol/l glutamine, 0.075% sodium bicarbonate, 0.1% non-essential amino acids, 2.5% fetal calf serum, 3% horse serum, 10% fresh rat serum, 1 nmol/l T_3 and 40 $\mu\text{g/l}$ dexamethasone, pH 7.3, to a density of 2×10^5 cells/ml. The cells were seeded onto microtitre plates (Nunc, Roskilde, Denmark), 200 $\mu\text{l/well}$, and cultured for 3 days at 37 °C and 8% CO_2 .

Following the culture period the cells were washed twice with stimulation buffer (HBSS supplemented with 1% BSA, 0.25% D-glucose and 25 mmol/l HEPES, pH 7.3) and preincubated for 1 h at 37 °C and 5% CO_2 . The buffer was exchanged with new stimulation buffer (37 °C). Test compound solution was added and the plates were incubated for 15 min at 37 °C and 5% CO_2 . The medium was decanted and analysed for released GH.

When testing the effect of antagonists the cells were incubated with antagonist (final concentration 1 nmol/l to 100 $\mu\text{mol/l}$) together with 10 nmol/l ipamorelin, GHRP-6 or 1 nmol/l GHRH. The medium was collected following a 15-min incubation period and analysed for GH content. All assays were performed in triplicate.

Effects in anaesthetised rats

The animals were purchased and housed under the same conditions as described above. Pentobarbital dissolved in 0.9% NaCl (final concentration 50 mg/ml) was administered i.p. (dose volume 1.2 ml/kg) to Sprague-Dawley male albino rats (250 ± 25 g). After 15 min, a blood sample of 0.4 ml was obtained from the orbital vein (sample 0). Immediately after blood sampling 2 ml/kg of a test compound solution was administered i.v. as a bolus injection through the tail vein. After 10 min, a new sample of 0.4 ml was obtained from the orbital vein (sample 10). The samples were collected in heparin-coated vials and kept on ice for 15–20 min prior to centrifugation at 1300 g for 3 min. Plasma was frozen and stored at -20 °C until the GH assay. At least five different doses of each compound were tested using six rats per dose level.

Effects in conscious swine

Six female, 40 kg slaughter swine of the breed Landrace Yorkshire cross (Lars Holmenlund, Denmark) were used for each compound. The swine were housed at the test facilities at least one week prior to the experiments.

Jugular catheters were inserted and fixed under general halothane anaesthesia at day zero. Ipamorelin, GHRP-2 and GHRP-6 were administered as i.v. bolus

injections in increasing doses with 24-h intervals in five doses per experiment. The dose range was at least a factor of 100. GHRH was administered at 6 nmol/kg (30 $\mu\text{g/kg}$) corresponding to 200–300 times the ED_{50} for GH release. The test compounds were dissolved in saline containing 0.5% porcine serum albumin and the dose volume was 0.02 ml/kg. Blood samples were drawn from the jugular catheter at frequent intervals from 30 min prior to stimulation until 3 h post stimulation. The blood was centrifuged at 1000 g for 10 min in heparin-coated tubes and the plasma stored at -80 °C until GH analysis.

Other hormones besides GH were measured using at least three different dose levels for the GHRP receptor-active compounds. The dose of GHRH was 6 nmol/kg. Blood samples were drawn from the jugular catheter at 0, 10, 30, 45, and 60 min after the bolus injection. The blood was stabilised with heparin and centrifuged at 1000 g for 10 min and stored at -80 °C until analysed for follicle-stimulating hormone (FSH), luteinizing hormone (LH), PRL, thyrotrophin (TSH), ACTH and cortisol.

In a control experiment a series of swine was dosed with isotonic saline. In this experiment no significant increases in the plasma hormone levels were observed. Thus, the average zero time plasma levels of ACTH and cortisol observed in the stimulation experiments were used as basal levels.

Hormone assays

The rat GH was measured by an in-house-developed competitive RIA using ^{125}I -labelled rat GH, rabbit antibody against rat GH and scintillation proximity assay particles (SPA-particles, Amersham International, Amersham, Bucks, UK) coated with antibody against rabbit antibody. The detection limit was 4 ng/ml plasma and the intra- and interassay coefficients of variation were 9.5% and 6.2% respectively.

The porcine (p) GH ELISA was a double monoclonal sandwich immunoassay using in-house-made antibodies prepared against pGH. The detection limit was 0.3 ng/ml plasma and the intra- and interassay coefficients of variation were 4.6% and 2.3% respectively.

ACTH was determined by a two-site immunoradiometric assay (IRMA), as described previously (20), using a commercial test kit (Euro-Diagnostica BV, Apeldoorn, The Netherlands). The sensitivity of the assay was 0.8 pg/ml plasma. The intra- and interassay coefficients of variation were 2.6 and 4.7% respectively. FSH and LH levels were measured by a double-antibody RIA as described by Van der Meulen (21) using porcine FSH and LH for standard and for iodination. The sensitivity of the FSH assay was 1.9 ng/ml plasma at the 90% B/B_0 level and that of LH was 0.13 ng/ml plasma. The intra- and interassay coefficients of variation were 8.2 and 11.0% and 10.6 and 13.8% respectively for the FSH and LH assays. PRL was analysed by RIA as previously described (22). The intra- and interassay coefficients of

variation were 6.9 and 12.3% respectively. TSH was measured using a double-antibody RIA developed by Dr F Helmond, The Netherlands (personal communication) using porcine TSH for standard and for iodination. The intra-assay coefficient of variation of this assay was 6.0%. Cortisol was measured directly in plasma samples using a single-antibody RIA technique as described by Janssens *et al.* (23). The sensitivity of the cortisol assay was 0.5 ng/ml plasma at 90% B/B₀. The intra- and interassay coefficients of variation were 4.5 and 11.2% respectively.

Calculations

The basal GH level for individual swine was calculated as the average of the three GH values obtained prior to stimulation. For other hormones the average value obtained at time zero immediately prior to stimulation was considered to be the basal value (see 'Effects in conscious swine' for details). Peak hormone levels adjusted for basal level (C_{\max}), obtained following administration of test compounds, the time of C_{\max} (T_{\max}), and the area under the time concentration curves (AUC) calculated using the trapezoidal rule, were used to characterize the hormone response of individual swine. Dose-response curves were constructed using peak GH levels for rats and C_{\max} GH plasma concentrations for swine. Fitting to the Hill equation or hyperbolic Michaelis-Menten equation was performed by nonlinear regression, from which the efficacy (maximal GH released, E_{\max}) was estimated. For the *in vitro* results the efficacy was expressed relative to the maximal GH released by GHRP-6.

Using the E_{\max} values of the individual compounds, the potency was calculated as the dose inducing half-maximal stimulation/inhibition ($EC_{50}/ED_{50}/IC_{50}$ values). In inhibition studies, the IC_{50} values were converted to inhibition constant (K_i) values using the Cheng-Prusoff equation (24).

Dose-response curves for all other pituitary hormones and for cortisol were constructed using the same procedure as for GH in swine.

The results were tested for normal probability distribution by the Shapiro-Wilk test. Non-parametric

Table 2 Pharmacological profile of ipamorelin-, GHRP-6- and GHRH-induced GH release in rat pituitary cells *in vitro* in the presence of GHRP and GHRH antagonists. The results are shown as means \pm S.E.M. ($n = 3-6$ separate experiments).

Secretagogue	K_i (nmol/l)			
	D-Lys ³ -GHRP-6	L-692,400	SPA	GHRH ant.
Ipamorelin	565 \pm 234	2346 \pm 481	20 \pm 4	>17 000
GHRP-6	1167 \pm 167	2500 \pm 674	15 \pm 7	>17 000
GHRH	>17 000	>10 000	>17 000	111 \pm 18

SPA, (D-Arg¹,D-Phe¹,D-Trp^{7,9},Leu¹¹)- Substance P; GHRH ant., (N-Acetyl-Tyr¹,D-Arg²)-hGHRH(1-29)NH₂.

tests were used for data with non-likely normal probability distribution. All statistical comparisons were performed using SAS (NC, USA) and Prism (Graphpad, CA, USA) software.

Results

Rat pituitary cell assay

All secretagogues stimulated GH release from rat pituitary cells in a dose-dependent manner. GHRP-1, GHRP-2 and GHRP-6 were approximately equipotent in this assay. When the central dipeptide Ala-Trp was deleted (NNC 26-0039) a slight decrease in EC_{50} but no effect on E_{\max} as compared with GHRP-1 was observed. By replacing the N-terminal L-Ala with D-Ala (NNC 26-0133) a more pronounced decrease in the potency and a slight decrease in E_{\max} was observed.

If L-Ala was replaced by Aib (ipamorelin) both the EC_{50} and E_{\max} values were restored to the level of GHRP-1 ($EC_{50} = 1.3 \pm 0.4$ nmol/l vs 1.1 ± 0.3 nmol/l and $E_{\max} = 85 \pm 5\%$ vs $80 \pm 5\%$). Details of these results can be seen in Table 1.

To evaluate the pharmacological specificity of ipamorelin we investigated whether the GHRP antagonists ((D-Lys³)-GHRP-6, L-692,400 and (D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹)-Substance P) or the GHRH antagonist (N-Acetyl-Tyr¹,D-Arg²)-hGHRH(1-29)NH₂ affected the stimulation. As evident from Table 2, the GHRP antagonists had no effect on GHRH-induced GH release whereas the GH releasing effects of both GHRP-6 and ipamorelin were inhibited with similar potencies. In contrast, the GHRH antagonist (N-Acetyl-Tyr¹,D-Arg²)-hGHRH(1-29)NH₂ potently inhibited GHRH-induced GH release, but had no effect on GH release induced by either GHRP-6 or ipamorelin. This clearly suggests a GHRP receptor agonist profile of ipamorelin.

Effects in anaesthetised rats

We evaluated the effect of some of these GH secretagogues in anaesthetised rats. The results, listed in Table 3,

Table 3 The effect of some novel pentapeptides on GH release *in vivo* in rat and swine - a comparison with GHRP-2 and GHRP-6. The results are shown as means \pm S.E.M. (from C_{\max} GH values), ($n = 6-18$ separate experiments).

Secretagogue	Rat		Swine	
	ED_{50} (nmol/kg)	E_{\max} (ng/ml)	ED_{50} (nmol/kg)	E_{\max} (ng/ml)
Ipamorelin	80 \pm 42	1545 \pm 250	2.3 \pm 0.03†	65 \pm 0.2
NNC 26-0039	>4300*	0	—	—
NNC 26-0133	186 \pm 126	587 \pm 93*	—	—
GHRP-2	30 \pm 4	1153 \pm 31	0.6 \pm 0.2	56 \pm 6
GHRP-6	115 \pm 36	1167 \pm 120	3.9 \pm 1.4†	74 \pm 7

* $P < 0.05$ vs all the secretagogues; † $P < 0.05$ vs GHRP-2 (unpaired *t*-test).

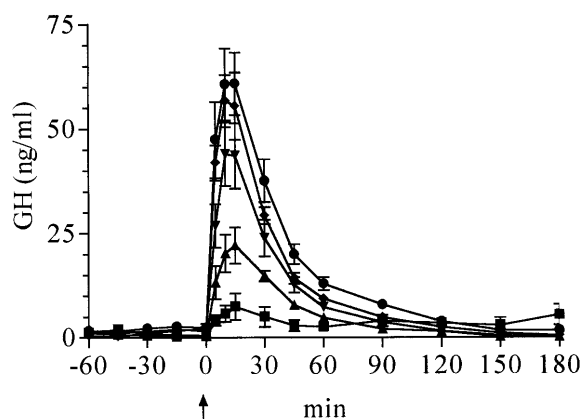


Figure 1 Plasma GH levels versus time in swine following i.v. administration of different doses of ipamorelin: (■) 0.4 nmol/kg; (▲) 1.4 nmol/kg; (▼) 4.2 nmol/kg; (◆) 14 nmol/kg; (●) 42 nmol/kg. Data, given as means \pm s.e.m., are from an experiment using six pigs at each dose and the basal level was subtracted from the stimulated values. See Materials and methods for experimental details. \uparrow indicates the time of dosing.

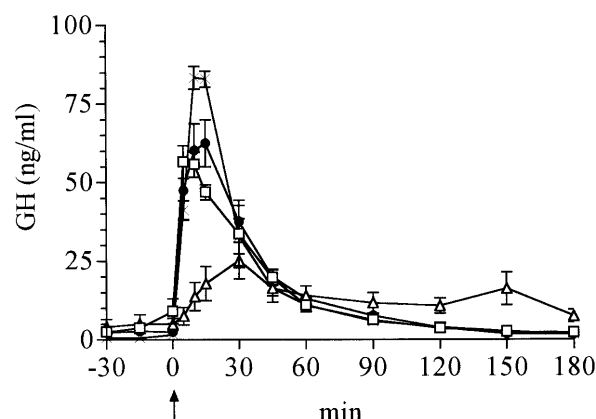


Figure 2 Plasma GH levels versus time in swine following i.v. administration of ipamorelin (●), GHRP-2 (□), GHRP-6 (x) and GHRH (Δ) using the most efficacious dose (see Table 4). Data, given as means \pm s.e.m., are from an experiment using six pigs at each dose and the basal level was subtracted from the stimulated values. For significance see Table 4. See Materials and methods for experimental details. \uparrow indicates the time of dosing.

demonstrated a clear discrepancy between *in vitro* and *in vivo*. Whereas only minor differences were observed *in vitro*, the *in vivo* results demonstrate that GHRP-2 was more than 100-fold more potent than NNC 26-0039 and four times more potent than GHRP-6. It was very surprising that NNC 26-0039, in spite of having high *in vitro* potency was inactive *in vivo*. NNC 26-0133, in which the L-Ala in NNC 26-0039 was substituted with D-Ala, regained most of its activity as compared with GHRP-6 ($ED_{50} = 186 \pm 126$ nmol/kg vs 115 ± 36 nmol/kg), but with a significantly lower E_{max} ($E_{max} = 587 \pm 93$ ng GH/ml plasma vs 1167 ± 120 ng GH/ml plasma). Ipamorelin, where the L-Ala in NNC 26-0133 was replaced by Aib, regained both potency and efficacy compared with GHRP-6 ($ED_{50} = 80$ nmol/kg ± 42 vs 115 ± 36 nmol/kg and $E_{max} = 1545 \pm 250$ ng GH/ml plasma vs 1167 ± 120 ng GH/ml plasma).

Effects in conscious swine

Ipamorelin, GHRP-2 and GHRP-6 all released GH in a dose-dependent manner. The peak GH response was

obtained within 15 min after stimulation and the GH plasma level returned to the basal level within 120 min. The plasma levels of GH versus time following various doses of ipamorelin are shown in Fig. 1. The ED_{50} and E_{max} values of all compounds are listed in Table 3. For the most efficacious dose of each compound and for GHRH at 6 nmol/kg, mean plasma GH versus time is shown in Fig. 2 and C_{max} , AUC and T_{max} values are listed in Table 4.

GHRP-2 ($ED_{50} = 0.6 \pm 0.2$ nmol/kg) was the most potent compound tested – more potent than both ipamorelin ($ED_{50} = 2.3 \pm 0.03$ nmol/kg) and GHRP-6 ($ED_{50} = 3.9 \pm 1.4$ nmol/kg). Stimulation with GHRP-6 caused the largest GH release in this study, with an E_{max} of 74 ± 7 ng GH/ml plasma. This was 12% higher than for ipamorelin and 25% higher than for GHRP-2. The AUC values for the three secretagogues demonstrated a similar picture as the C_{max} values.

Stimulation with GHRH (6 nmol/kg) induced GH release with a C_{max} of 22 ± 4 ng GH/ml plasma, significantly lower than found for GHRP-6, ipamorelin and GHRP-2. The GHRH response reached a peak approximately 40 min after the GHRH injection. A second, presumably endogenous peak was observed after 150 min. Even though GHRH had a C_{max} less than half that of GHRP-2, similar AUC values were found for these two compounds.

To study the specificity of the GH secretagogues, we evaluated the plasma levels of a number of other pituitary hormones (ACTH, FSH, LH, PRL, TSH) and cortisol.

None of the GH secretagogues tested affected plasma levels of FSH, LH, PRL or TSH in any of the administered doses. Mean basal levels were not significantly different from the C_{max} values obtained after stimulation ($P > 0.05$) (Table 5).

Table 4 The effect of different GH secretagogues in swine on plasma C_{max} , AUC and T_{max} . The results are shown as means \pm s.e.m. ($n = 6-18$).

Secretagogue	C_{max} (ng/ml)	AUC (ng \times min/ml)	T_{max} (min)
Ipamorelin (40 nmol/kg)	$63 \pm 8^*$	2354 ± 300	12 ± 1
GHRP-2 (40 nmol/kg)	$56 \pm 6^*$	$1660 \pm 286^*$	9 ± 4
GHRP-6 (70 nmol/kg)	84 ± 3	2687 ± 244	13 ± 1
GHRH (6 nmol/kg)	$22 \pm 4^{**}$	$1676 \pm 286^*$	$42 \pm 12^*$

* $P < 0.05$ vs GHRP-6; ** $P < 0.001$ vs ipamorelin, GHRP-2 and GHRP-6.

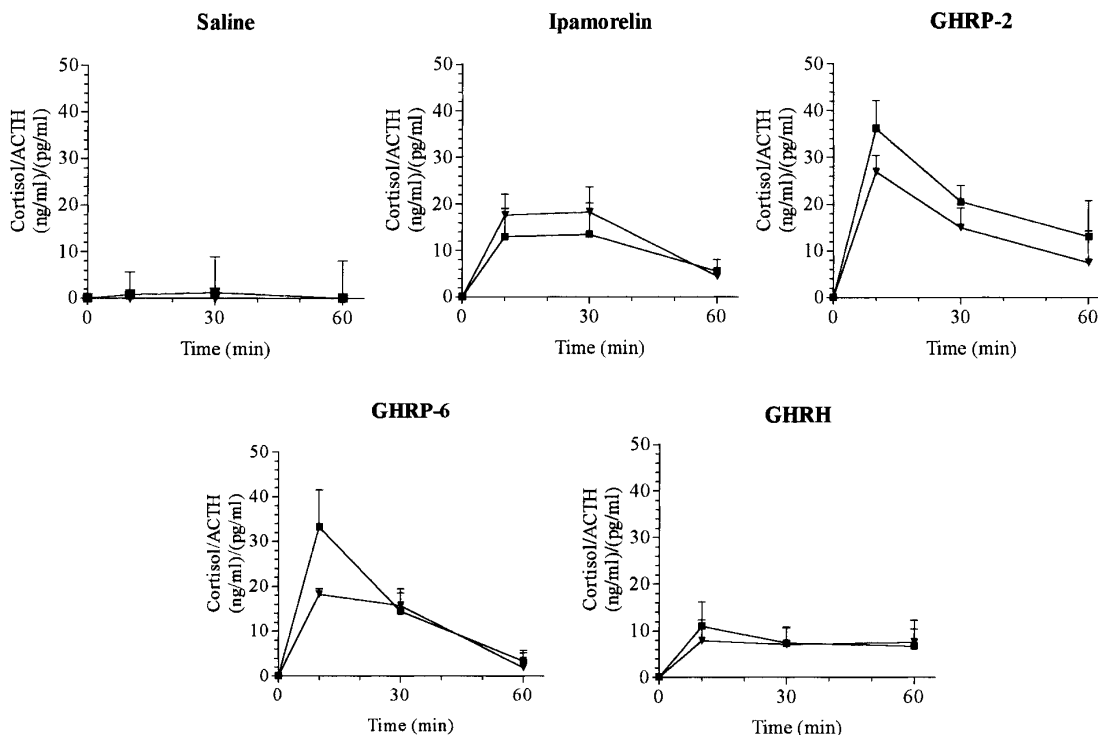


Figure 3 Plasma ACTH (■) and cortisol (▼) levels versus time in swine following i.v. administration of saline, ipamorelin (210×GH ED₅₀ dose), GHRP-2 (45×GH ED₅₀ dose), GHRP-6 (85×GH ED₅₀ dose) and GHRH (200–300×GH ED₅₀ dose). Data, given as means±S.E.M., are from a single experiment carried out in six pigs and the individual basal hormone level is subtracted from the stimulated values. See Materials and methods for experimental details. C_{max} values of ACTH and cortisol for GHRP-2 and GHRP-6 are significantly higher than those for ipamorelin and GHRH (*P*<0.05, unpaired *t*-test).

As ACTH and cortisol are associated with stress, plasma samples of these hormones often have a high degree of variability. To minimize the variation, all experiments were performed at the same time of the day, starting at 0900 h. The mean basal plasma level of ACTH was 15.6±1.1 pg/ml (mean±S.E.M., *n*=58, range 7.8–49.7 pg/ml) and of cortisol was 11.9±1.1 ng/ml (mean±S.E.M., *n*=56, range 1.0–37.0 ng/ml).

In Fig. 3, data are shown of the time dependency from a single experiment evaluating ACTH and cortisol plasma levels before and after i.v. injection of saline, ipamorelin, GHRP-2, GHRP-6 and GHRH. The saline

challenge caused no significant increase in ACTH and cortisol plasma levels. GHRH showed a slight increase in both ACTH and cortisol plasma levels, at a dose 200- to 300-fold higher than the ED₅₀ for inducing GH release (6 nmol/kg). On the other hand, both GHRP-6 and to an even greater extent GHRP-2 induced large and significant increases in plasma ACTH and cortisol levels. The effect of GHRP-6 and GHRP-2 was maximal at doses 85- and 45-fold higher than the ED₅₀ for releasing GH.

Very surprisingly, ipamorelin, even at doses more than 200-fold higher than the ED₅₀ value for GH release, showed very limited effects on ACTH or cortisol

Table 5 The effect of ipamorelin (420 nmol/kg), GHRP-2 (270 nmol/kg), and GHRP-6 (340 nmol/kg) in swine on plasma levels of FSH, LH, PRL and TSH. The results are shown as means ± S.E.M. (*n*=6).

Secretagogue	FSH (ng/ml)		LH (ng/ml)		PRL (ng/ml)		TSH (ng/ml)	
	Basal	C _{max}	Basal	C _{max}	Basal	C _{max}	Basal	C _{max}
Ipamorelin	26.4 ± 6.5	35.9 ± 6.2	5.0 ± 0.6	4.6 ± 0.4	5.1 ± 0.1	5.7 ± 0.2	7.1 ± 1.4	7.3 ± 1.7
GHRP-2	29.3 ± 3.9	33.2 ± 5.1	3.6 ± 0.3	3.7 ± 0.3	5.2 ± 0.3	5.7 ± 0.2	8.4 ± 0.5	8.9 ± 0.5
GHRP-6	26.6 ± 4.6	27.4 ± 4.0	3.7 ± 0.5	3.9 ± 0.4	5.1 ± 0.5	6.2 ± 0.6	8.5 ± 0.7	8.7 ± 1.0

No significant differences between basal and C_{max} were obtained.

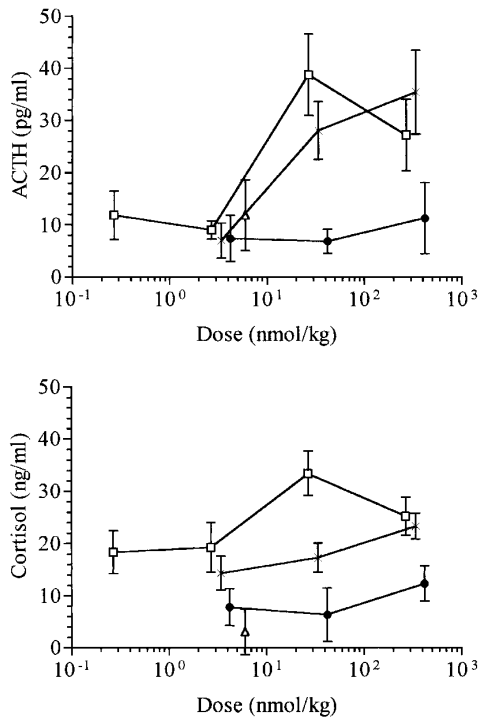


Figure 4 Plasma ACTH and cortisol following i.v. administration of increasing doses of ipamorelin (●), GHRP-2 (□) and GHRP-6 (x). As a reference the corresponding value obtained with GHRH (6 nmol/kg, (△)) is noted in the Figure. Data are given as means \pm s.e.m. and are average values from 3–4 separate experiments using six pigs in each experiment. The average basal hormone level (ACTH, 15.8 pg/ml; cortisol, 11.9 ng/ml) has been subtracted from the stimulated values. See Materials and methods for further experimental details. Both GHRP-2 and GHRP-6 display a significant dose–response relationship with ACTH ($P < 0.004$ and $P < 0.007$, unpaired *t*-test) and cortisol ($P < 0.03$ and $P < 0.08$ respectively, unpaired *t*-test) plasma levels.

plasma levels. The effect of ipamorelin was at no point significantly different from the effect of GHRH on these hormones.

Average data of the dose–response relationships for ipamorelin, GHRP-2 and GHRP-6 on ACTH and cortisol plasma levels are shown in Fig. 4. Evidently, a clear dose–response relationship exists for GHRP-2 and GHRP-6, but not for ipamorelin.

No signs of adverse effects were observed with any of the secretagogues at the doses tested.

Discussion

In this paper we present ipamorelin, the first GHRP receptor-active compound with selectivity for GH release.

A number of GHRP receptor-active compounds have previously been described as releasing GH in several species, including rats (10), dogs (11), swine (12) and man (13–16). In our work on identifying novel small molecules with GH releasing properties, we

hypothesised based on previous work (25) and a superposition of GHRP-1 and L-692,429, that the central dipeptide Ala-Trp of GHRP-1 may be redundant (see Fig. 5).

When the central Ala-Trp sequence was deleted from GHRP-1 (NNC 26–0039), we found the *in vitro* potency to be similar to that of GHRP-1. The relatively high potency found *in vitro* led us to investigate NNC 26–0039 in male rats to elucidate its *in vivo* effect on GH release. Surprisingly however, even at doses of 4300 nmol NNC 26–0039/kg no GH release was observed. By inverting the stereochemistry at the NNC 26–0039 N-terminal from L-Ala to D-Ala (NNC 26–0133) the *in vivo* activity in rats was regained. This observation led us to study further the apparently crucial role of the N-terminal, and we combined the two previous N-terminals (L- and D-Ala) to Aib (ipamorelin), and observed not only an increase in the *in vitro* activity but also in the *in vivo* activity.

To demonstrate that ipamorelin was acting on a GHRP-like receptor the pharmacological mechanism of action was investigated *in vitro*, using previously described GH secretagogue antagonists. GHRP-6- and ipamorelin-induced GH release was inhibited with similar potencies by the GHRP receptor antagonists (D-Lys³)-GHRP-6 (26), L-692,400 (27) and (D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹)-Substance P (27). On the other hand, the GHRH antagonist (N-Acetyl-Tyr¹,D-Arg²)-hGHRH(1–29) (28) had no effect on the GHRP-6- and ipamorelin-induced GH release. As expected, GHRH-induced GH release demonstrated the opposite pharmacological profile. These results strongly suggest that ipamorelin, like GHRP-6, mediates its effect through a GHRP-like receptor – a receptor distinct from the GHRH receptor.

These initial promising results with ipamorelin encouraged us to investigate further this compound as a novel GH secretagogue in swine. The swine seems to be a suitable animal model because of its close similarity to humans in several physiological aspects (29). Swine have an endogenous GH profile comparable to humans with respect to basal GH levels, little sex dimorphism, GH pulse frequency and amplitudes (30). The only major difference between swine and humans in the GH secretion pattern is that swine lack a major nocturnal GH pulse – probably due to the difference in sleeping patterns.

In the present studies, the acute effects of the GH secretagogues ipamorelin, GHRP-2 and GHRP-6 were evaluated in conscious swine. To study the specificity, the possible release of other anterior pituitary hormones (ACTH, FSH, LH, PRL, and TSH) and cortisol was also examined. The effects of the GH secretagogues were compared with that of GHRH, as this was considered to be the optimal physiological reference GH secretagogue.

In this porcine model, test compounds were given as a bolus injection and the plasma GH levels were monitored for three hours. The study was designed as an open non-balanced dose-escalation study. The intervals between doses, however, were chosen to be

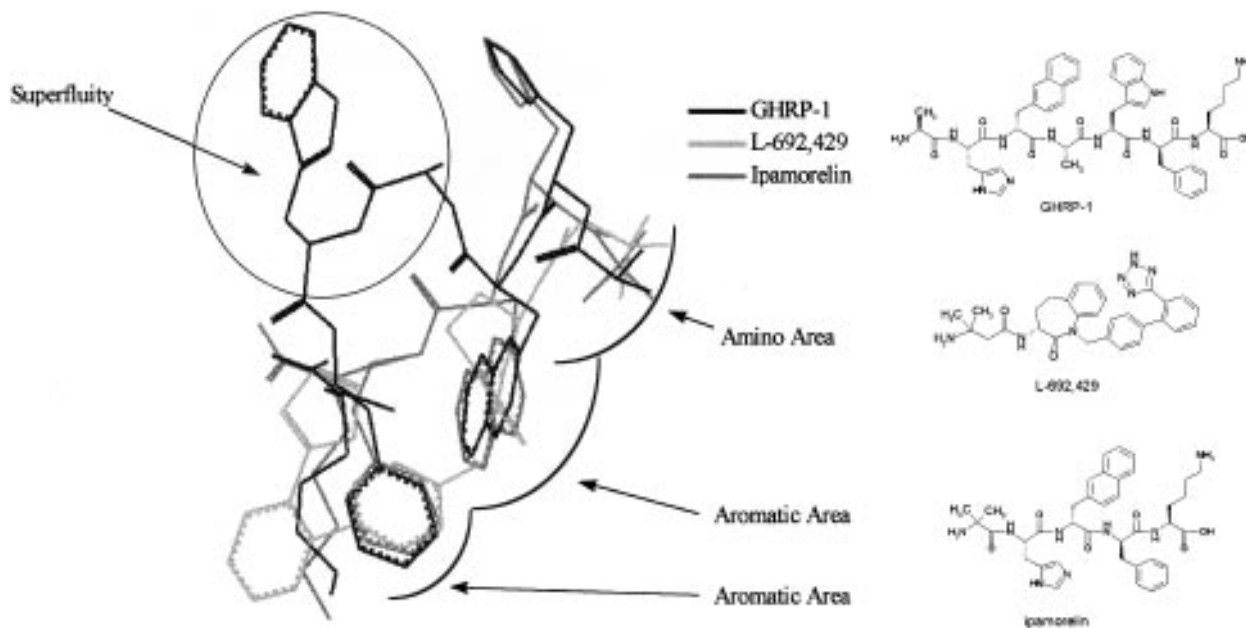


Figure 5 Superposition of GHRP-1, L-692,429 and ipamorelin. The primary amino group in the N-terminal of GHRP-1, L-692,429 and ipamorelin, the aromatic moiety of D-Nal of GHRP-1 and ipamorelin and the benzolactam moiety of L-692,429, and the aromatic moiety of D-Phe of GHRP-1 and ipamorelin and the biaryl moiety of L-692,429 have been overlapped.

24 h, much longer than the plasma half-life of any of the three secretagogues tested (unpublished observations). In a pilot study, using this time interval between doses, we found no variation in C_{max} or AUC when GHRP-6 (20 nmol/kg) was administered repeatedly six times in six swine (31). Therefore, the risk of introducing carry-over effects in the study was considered negligible.

All three compounds gave rise to an immediate GH release in a dose-dependent manner, GHRP-2 being the most potent with an ED_{50} of 0.6 ± 0.2 nmol/kg. This is in agreement with the *in vivo* rat results and also as described for humans (14). GHRP-6 had the highest efficacy with an E_{max} of 74 ± 7 ng GH/ml plasma. The novel secretagogue ipamorelin had an E_{max} of 90% of the level of GHRP-6, with a peak response eliciting a 20- to 30-fold increase in basal GH levels. Ipamorelin was slightly more potent than GHRP-6, but less potent than GHRP-2. The time for peak response (T_{max}) was around 10 min, in agreement with other GH secretagogues in different species, including man (12, 32–34).

The GH release in swine after maximal GHRH stimulation (6 nmol/kg) results in a C_{max} of 22 ± 4 ng GH/ml plasma. This is 3- to 4-fold lower than following stimulation with the most efficacious doses of the GHRP receptor-active GH secretagogues. This is in agreement with previous observations in swine (12) and humans (34). GHRH gave rise to GH release with a delayed T_{max} (Table 4) compared with that of GHRPs, also in agreement with previously published results for swine and humans (12, 34). Although GHRH stimulation exhibits a lower C_{max} – less than half that of GHRP-2 – the protracted profile and additional endogenous GH

pulse cause a relatively greater AUC, resulting in nearly identical AUCs for GHRH and GHRP-2.

All secretagogues tested were able to release GH without affecting FSH, LH, PRL and TSH. This is in agreement with results described in swine with respect to LH (32) and those described in beagle dogs for PRL (33), but is in contrast to that found in humans after hexarelin stimulation with respect to PRL (35). This might be explained by a different level of mammosomatotrophs in various species. Humans have a high proportion of these cells, which are an intermediate form between mammatrophs secreting PRL and somatotrophs secreting GH (36). Furthermore, it has been found in rats that the numbers of mammosomatotrophs increase markedly with age (37). Using young swine in this study might be part of the reason for not observing any PRL release.

In vivo experiments describing ACTH and cortisol plasma levels are often impaired by a large variation, presumably due to association of these two hormones to stress. In this study design, a saline challenge had no significant effect on the basal plasma levels of these two hormones. When testing GHRH, we observed a slight increase in both ACTH and cortisol. The finding that GHRH is not totally specific with respect to GH release is in agreement with previously described studies in both swine and humans (12, 38). This slight effect on the corticotroph system might be an indirect effect of GHRH on other neurons involved in the hypothalamic–pituitary–adrenal axis. GHRP-6, and to an even greater extent GHRP-2, dose-dependently increased both ACTH and cortisol in a somewhat parallel fashion. The

increase in plasma levels of cortisol stimulated by GHRP-2 in this study corresponds to the levels achieved when stimulating swine with 150 µg corticotrophin releasing hormone (CRH) (12). Also in humans it has been shown that GHRP-2 increases the plasma level of cortisol similar to that shown with CRH (17). Administration of ipamorelin, like GHRH, caused an increase in the plasma levels of both ACTH and cortisol. However, to our surprise ipamorelin – even in extreme doses – was unable to increase ACTH or cortisol plasma concentrations to a level significantly different from those induced by GHRH. Also, ipamorelin showed no dose-dependent effects on either ACTH or cortisol plasma levels, contrary to the situation with GHRP-2 and GHRP-6.

These findings are very interesting. First, they show that GHRP-2 and GHRP-6 dose-dependently cause an increase in both ACTH and cortisol plasma levels. Noticeably, GHRP-2 and GHRP-6 produced an ACTH release of 350% ($P < 0.05$) and 300% ($P = 0.06$) respectively, of GHRH, although they were administered in doses relatively closer to the respective ED₅₀ values for GH release than were GHRH and ipamorelin. Consequently, both GHRP-2 and GHRP-6 cannot be considered as selective GH secretagogues. Secondly, ipamorelin acts, like GHRH, on the plasma levels of either hormone. Consequently, ipamorelin is the first GHRP receptor-active GH secretagogue, with selection for GH release which does not differ from that of GHRH. Thirdly, the finding that increase in plasma ACTH was always followed by a correlated increase in plasma cortisol suggests that the effect of the GHRPs on cortisol is mediated via ACTH release. This mechanism of action is in agreement with previous findings in swine where the effect on cortisol is abolished after disconnection of the hypothalamus stalk (12).

Introduction of GHRP receptor-active GH secretagogues with selectivity for GH release will be advantageous to the patient. Although this study did not include human data, the results found in swine indicate that ipamorelin and other selective GH secretagogues might be expected to be superior to other GH secretagogues currently used in clinical trials.

In summary, we have characterised the novel GH secretagogue ipamorelin *in vitro* and *in vivo*. *In vitro*, ipamorelin displays a typical GHRP-6-like GHRP receptor agonist profile. However, *in vivo* ipamorelin had a very unique profile being the first GHRP receptor-active GH secretagogue with specificity for GH release. Consequently, ipamorelin and related secretagogues hold great promise for the future.

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