Abstract

NN703 is a novel orally active GH secretagogue (GHS) derived from ipamorelin. NN703 stimulates GH release from rat pituitary cells in a dose-dependent manner with a potency and efficacy similar to that of GHRP-6. The effect is inhibited by known GHS antagonists, but not by a GH-releasing hormone antagonist.

Binding of 35S-MK677 to the human type 1A GHS receptor (GHS-R 1A) stably expressed on BHK cells was inhibited by GHRP-6 and MK677 as expected. NN703 was also able to inhibit the binding of 35S-MK677. However, the observed K_i value was lower than expected, as based on the observed potencies regarding GH release from rat pituitary cells. Similarly, the effect of NN703 on the GHS-R 1A-induced inositol phosphate turnover in these cells showed a lower potency, when compared with GHRP-6 and MK677, than that observed in rat pituitary cells.

The effect of i.v. administration of NN703 on GH and cortisol release was studied in swine. The potency and efficacy of NN703 on GH release were determined to be 155 ± 23 nmol/kg and 91 ± 7 ng GH/ml plasma respectively. A 50% increase of cortisol, compared with basal levels, was observed for all the tested doses of NN703, but no dose-dependency was shown.

The effect of NN703 on GH release after i.v. and oral dosing in beagle dogs was studied. NN703 dose-dependently increased the GH release after oral administration. At the highest dose (20 μmol/kg), a 35-fold increase in peak GH concentration was observed (49.5 ± 17.8 ng/ml, mean ± s.e.m.). After a single i.v. dose of 1 μmol/kg the peak GH plasma concentration was elevated to 38.5 ± 19.6 ng/ml (mean ± s.e.m.) approximately 30 min after dosing and returned to basal level after 360 min. The oral bioavailability was 30%. The plasma half-life of NN703 was 4.1 ± 0.4 h.

A long-term biological effect of NN703 was demonstrated in a rat study, where the body weight gain was measured during a 14-day once daily oral challenge with 100 mmol/kg. The body weight gain was significantly increased after 14 days as compared with a vehicle-treated group.

In summary, we here describe an orally active and GH specific secretagogue, NN703. This compound acts through a similar mechanism as GHRP-6, but has a different receptor pharmacology. NN703 induced GH release in both swine and dogs after i.v. and/or p.o. administration, had a high degree of GH specificity in swine and significantly increased the body weight gain in rats.

Introduction

Bowers et al. (1) reported in 1977 the discovery of a series of small peptides which stimulated the release of growth hormone (GH) from pituitary cells by a different mechanism of action than GH-releasing hormone (GHRH). The prototype of this new class of GH secretagogues (GHS), GHRP-6 (His-d-Trp-Ala-Trp-o-Phe-Lys-NH_2), has, when given by parenteral route, shown to be highly potent in several animal species including humans (2–4).

The discovery of GHSs might improve the possibility for treatment of disorders related to GH deficiency. The advantage of the GHSs is their ability to amplify endogenous pulsatile GH secretion, while maintaining normal feedback mechanisms. Another important effect of the GHSs is the ability to restore serum insulin-like growth factor-I (IGF-I) in elderly adults to concentrations similar to those of young adults (5).

A specific animal and human GHS receptor type 1A (GHS-R 1A) with high affinity for MK677, a non-peptidyl GHS, has recently been cloned (6). This receptor is present in the pituitary, hypothalamus and other areas of the central nervous system (7), and it is strongly believed that the GHSs mimic a putative endogenous ligand which plays a critical role in the regulation
of GH secretion and in certain extra neuroendocrine activities such as sleep (8) and food intake (9, 10).

Since the discovery of GHRP-6 (11) and its analogues, a number of new chemical entities with similar characteristics have been discovered. These include both peptides (GHRP-1, GHRP-2 (12), GHRP-6 (11), hexarelin (13), ipamorelin (14, 15)) and non-peptides (L-692,429 (16) and MK677 (17)). Of these only MK677 has proven good oral bioavailability.

With the intention to make a new orally active GHS we used ipamorelin as template and by using a peptidomimetic strategy we obtained several new orally active GHSs (14, 18). From this series we now report a novel GHS, NN703, derived from ipamorelin with good in vivo potency, high specificity and improved oral bioavailability.

Materials and methods

Chemistry

NN703 ((2E)-5-amino-5-methylhex-2-enoic acid N-methyl-N-((1R)-1-(N-methyl-N-((1R)-1-(methylcarbamoyl)-2-phenylethyl)carbamoyl)-2-(2-naphthyl)ethyl)amide) (Fig 1) is a modified tripeptide derived from ipamorelin (15), via a peptidomimetic approach as previously described by Hansen et al. (18).

Rat pituitary cell assay

All media were obtained from Life Technologies (Paisley, Strathclyde, UK), trypsin from Worthington (NJ, USA), bovine serum albumin (BSA), DNase, tri-iodothyronine (Sigma, St Louis, MO, USA). All media were obtained from Life Technologies (Paisley, Strathclyde, UK), trypsin from Worthington (NJ, USA), bovine serum albumin (BSA), DNase, tri-iodothyronine (Sigma, St Louis, MO, USA). Rat pituitary cell assay

Cultures of pituitary cells were prepared according to Raun et al. (15). Briefly, pituitary cells isolated from Sprague–Dawley male rats (250 ± 25 g) were cultured for 3 days. Following the culture period the cells were incubated 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% CO₂. New stimulation buffer and test compound was added and after 15-min incubation at 37°C and 5% CO₂ the medium was decanted and analysed for GH.

When testing the antagonist effects, the cells were incubated with antagonist (final concentration 1 nmol/l to 100 nmol/l) together with 50 nmol/l NN703, 10 nmol/l 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.

Receptor binding assays

Isolated crude membranes from BHK cells, stably expressing the human GHS-R1A, were suspended in homogenising buffer (2.5 mmol/l Tris–base, 2.5 mmol/l EDTA, 10 mmol/l MgCl₂ and 30 μg/ml bacitracin) to a concentration of 0.5 mg protein/ml and added to microtitre plates to a final total concentration of 50 μg protein/ml together with ³⁵S-MK677 (40 000 c.p.m./well) and with or without different concentrations (0.001 nmol/l to 10 μmol/l) of different GHSs and binding buffer (2.5 mmol/l Tris–base, 2.5 mmol/l EDTA and 10 mmol/l MgCl₂) up to a total volume of 250 μl. Non-specific binding was obtained by adding 10 μmol/l MK677. The membranes were incubated at 30°C for 30 min, and bound radioligand was separated from free by washing with binding buffer through GF/B filters (Whatman, Maidstone, Kent, UK) pretreated with 0.5% polyethylenimine for 60 min. The radioactivity on the filters was counted in Optiphase HiSafe 3 (Wallac, Turku, Finland).

Binding experiments to crude cell membranes prepared from transiently transfected COS-7 cells were performed as described by Howard et al. (6). All tested compounds displaced the radioactive ligand to the level of non-specific binding.

Pharmacodynamic studies in swine

Six female Danish slaughter swine (40–50 kg) of the breed Landrace Yorkshire cross (Lars Holmlund, Denmark) were used. The swine were housed at the test facilities 3 weeks prior to the experiments. They were treated according to Raun et al. (15).

Briefly, NN703, hexarelin, MK677 or vehicle were administered as single i.v. injections over 20 s (immediately after time 0), in increasing doses with 72-h intervals from day 2 and onwards in six doses (from 0.1 to 10 000 nmol/kg). Frequent blood samples were drawn between 30 min and 180 min. Plasma was analysed for porcine GH (pGH).

The release of cortisol was examined at three of the six dose levels: 100, 1000 and 10 000 nmol/kg NN703 and MK677 and 3, 30 and 300 nmol/kg hexarelin. Blood samples were drawn from the jugular catheter at the times −30, 0, 10, 30, 60 and 120 min and stored at −20°C until analysis.

Pharmacokinetic and pharmacodynamic studies in dogs

Oral bioavailability studies were conducted in male and female beagle dogs. The dogs were fasted overnight prior
to dosing. Diet was withheld for at least 3 h post dosing. A 1-week wash-out period separated p.o. and i.v. dosing. NN703 was administered in a vehicle of citrate/phosphate buffer, pH 5.0. For p.o. administration the dogs received a dose of 1, 5, 20 μmol NN703/kg by stomach tubing. For i.v. administration the dogs received a dose of 1 μmol NN703/kg as a bolus in a hind leg vein. EDTA blood samples were drawn from a front leg vein at intervals up to 23 h after dosing. Blood samples were placed on an ice-water bath immediately after sampling. Plasma was separated by centrifugation and stored frozen pending analysis for canine GH (cGH) and NN703.

**Body weight gain in rats**

Sprague–Dawley female rats of 5 months of age were obtained from the Møllegaard Breeding Centre (DK-4620, Denmark). They were housed in cages (two animals/cage) under standardised humidity and temperature conditions with 12-h light cycles. They were fed a standard rat chow and had free access to drinking water. The rats were randomly allocated by block randomisation according to weight to two treatment groups each containing 16 animals. They were dosed with 100 μmol/kg NN703. Each rat was dosed once daily for 14 days. The doses of either NN703 or vehicle were applied in the morning by oral gavage using a rubber catheter and a fixed dosing volume of 1 ml. The rats were weighed every morning just before dosing.

Twenty-four hours after the last dosing, 12 rats from each treatment group were anaesthetised with pentobarbital (60 mg/kg i.p.) and fitted with catheters in the carotid artery and the jugular vein. NN703 at a concentration of 3 μmol/kg was applied as a bolus i.v. injection in a volume of 0.5 ml/kg at time 0. Blood samples of 400 μl were drawn before and at 5, 10, 15, 20 and 30 min after dosing. After 45 min at room temperature, the blood samples were centrifuged and the serum was isolated. The samples were kept at −20°C until analysis for rat GH and the sample at time 0 for IGF-I.

**Calculations**

**In vitro** For the GH-releasing results, the efficacy (E_{max}) was expressed relative to the maximal GH release stimulated by GHRP-6. For the receptor binding studies, specific binding was defined as the difference between total binding and non-specific binding defined as ^{35}S-MK677 binding in the absence and presence of 10 μmol/l unlabelled MK677 respectively. Displacement curves were constructed using non-linear regression. Using the E_{max} values of the individual compounds, the potency was calculated as the dose inducing half-maximal stimulation/inhibition (EC_{50}/IC_{50} values). The IC_{50} values in the antagonist experiments as well as in the receptor experiments, were converted to K_i values using the Cheng–Prusoff equation (19).

**In vivo** The basal GH level for the individual swine was calculated as the average of the three plasma GH values obtained prior to stimulation.

The basal cortisol levels for the individual swine were calculated as the average of the two hormone values obtained prior to stimulation.

Peak hormone levels adjusted for basal level (C_{max}), obtained following administration of vehicle or test compounds, the time of C_{max} (T_{max}), and area under the concentration time curve (AUC) calculated by use of the trapezoidal rule were used to characterise the hormone response of individual swine. Dose–response curves were constructed using the C_{max} for GH plasma concentrations. For each dose the results were tested for normal probability distribution by the Shapiro–Wilk test. The mean was tested to be significantly different from zero (P < 0.05), by using a t-test for data with a likely normal probability distribution and a Mann–Whitney U test for data with non-likely normal probability distribution. If two or more of the doses were significantly different from zero and an analysis of variance showed significantly different C_{max} response between the doses, E_{max} and ED_{50} were estimated by fitting by non-linear regression. Cortisol release was expressed relative to basal levels for each swine in each dose.

All statistical procedures were performed using SAS (Gary, NC, USA) or Prism (GraphPad, CA, USA) software.

The oral bioavailability (f) was calculated as the total area under the plasma concentration versus time-curve following p.o. administration divided by the area following i.v. administration, appropriately corrected for dose. The AUC (0-tn) is the area under the plasma concentration curve from time zero to the last measurable concentration estimated by the trapezoidal rule. A_{t} is the terminal elimination rate constant, calculated by log-linear regression.

The systemic clearance (CL) is: CL = dose_{i.v.} / AUC_{i.v.} and the volume of distribution during the terminal phase (V_{d}) is: V_{d} = dose_{i.v.} / (AUC_{i.v.} · A_{t}).

**Analysis**

Rat and porcine GH and cortisol were measured as described in Raun et al. (15). cGH was measured in the pGH assay as cGH has an amino acid sequence identical to pGH. Recovery experiments at three levels were performed in dog plasma as well as checking linear dilution of dog GH samples.

The IGF-I concentration was determined based on acid–ethanol extraction using a commercial kit (Nichols Institute, CA, USA). Both the intra- and interassay coefficients of variation were below 10% with a limit of quantitation (LOQ) of 13.5 ng/ml. Inositol phosphate
(IP$_3$) was measured using D-myo-inositol 1,4,5-triphosphate $^3$H assay (TRK, 100, Amersham, UK).

Plasma concentrations of NN703 were determined after solid phase extraction by a reversed-phase HPLC method with uv detection (225 nm).

**Results**

**Rat pituitary cell assay**

NN703 stimulated GH release from primary rat somatotrophs with a potency of 2.7 ± 1.4 nmol/l (mean ± s.e.m., $n = 4$), similar to that of GHRP-6 and hexarelin, but less potent than MK677 (see Table 1). All the GHSs were equal in efficacy to GHRP-6.

To evaluate the pharmacological specificity of NN703 we investigated whether the GHRP antagonists ([D-Lys$^3$]-GHRP-6, L-692,400 and [D-Arg$^1$,D-Phe$^5$,D-Trp$^{7,9}$,Leu$^{11}$]-substance P) or the GHRH antagonist [N-acetyl-Tyr$^1$,D-Arg$^2$]-hGHRH (1–29)NH$_2$ affected the stimulation. As is 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 NN703 were inhibited by the GHRP antagonists with similar $K_i$ values. In contrast, the GHRH antagonist [N-acetyl-Tyr$^1$,D-Arg$^2$]-hGHRRH (1–29)NH$_2$ potently inhibited GHRH-induced GH release but had no effect on GH release induced by either GHRP-6 or NN703. This clearly suggests an in vitro pharmacological profile of NN703 similar to GHRP-6.

We further evaluated the specificity of NN703, using binding of $^{35}$S-MK677 to the human GHS-R 1A either stably expressed in a BHK cell line or transiently in COS-7 cells. These experiments demonstrated that GHRP-6, hexarelin and MK677, as expected, were able to inhibit specific $^{35}$S-MK677 binding in a rank order comparable to that found in the rat pituitary cell assay (Table 1 and Fig 2). NN703 also inhibited the binding of the radioactive ligand specifically (Fig. 2); however, this inhibition occurred with lower potency as compared with GHRP-6, hexarelin and MK677, as would be expected from the rat pituitary cell experiment (Table 2).

**Swine studies**

Intravenous administration of NN703 induced GH release in a dose-dependent manner. The plasma GH dose–response curves for NN703, MK677 and hexarelin are shown in Fig. 4. The potency and efficacy of NN703 was determined to be 155 ± 23 nmol/kg and 91 ± 7 ng GH/ml plasma (means ± s.e.m., $n = 6$) respectively. MK677 and hexarelin showed potencies and efficacies of 46 ±

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**Table 1** Comparison of biological activities and binding affinities in rat pituitary cells, and in BHK and COS-7 cells expressing the human GHS-R 1A. All results are expressed as means ± s.e.m. ($n = 3–12$).

<table>
<thead>
<tr>
<th></th>
<th>COS-7 membranes $K_i$ (nmol/l)</th>
<th>BHK membranes $K_i$ (nmol/l)</th>
<th>BHK cells $EC_{50}$ (nmol/l)</th>
<th>Rat pituitary cells $EC_{50}$ (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NN703</td>
<td>50.0 ± 2.1</td>
<td>384.0 ± 82</td>
<td>8.4 ± 2.9</td>
<td>2.7 ± 1.4</td>
</tr>
<tr>
<td>GHRP-2</td>
<td>0.58 ± 0.21*</td>
<td>nd</td>
<td>nd</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>GHRP-6</td>
<td>0.91 ± 0.43*</td>
<td>10.6 ± 2.6*</td>
<td>1.2 ± 0.1*</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Hexarelin</td>
<td>1.8 ± 0.3*</td>
<td>nd</td>
<td>nd</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Ipamorelin</td>
<td>63.4 ± 4.5</td>
<td>440 ± 216</td>
<td>46.9 ± 2.7*</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>MK677</td>
<td>0.29 ± 0.07*</td>
<td>0.46 ± 0.14*</td>
<td>2.4 ± 0.3*</td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>

* $P < 0.05$ compared with NN703.

nd, not determined. IP$_3$, inositol tris phosphate.

**Table 2** Pharmacological profile of NN703-, GHRP-6- and GHRH-induced GH release in rat pituitary cells in vitro. The results are shown as means ± s.e.m. ($n = 4$ separate experiments).

<table>
<thead>
<tr>
<th></th>
<th>o-Lys$^3$-GHRP-6 $K_i$ (nmol/l)</th>
<th>L-692,400 $K_i$ (nmol/l)</th>
<th>SPA $K_i$ (nmol/l)</th>
<th>GHRH antagonist $K_i$ (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NN703</td>
<td>885 ± 523</td>
<td>785 ± 176</td>
<td>3.1 ± 1.4</td>
<td>&gt; 17000</td>
</tr>
<tr>
<td>GHRP-6</td>
<td>1667 ± 357</td>
<td>2213 ± 560</td>
<td>8.0 ± 3.2</td>
<td>&gt; 17000</td>
</tr>
<tr>
<td>GHRH</td>
<td>&gt; 17000</td>
<td>&gt; 10000</td>
<td>&gt; 17000</td>
<td>111 ± 18</td>
</tr>
</tbody>
</table>

SPA = [o-Arg$^1$,o-Phe$^5$,o-Trp$^{7,9}$,Leu$^{11}$]-substance P; GHRH antagonist = [N-acetyl-Tyr$^1$,o-Arg$^2$]-hGHRRH (1–29)NH$_2$. 
6 nmol/kg and 121 ± 8 ng GH/ml plasma and 2.0 ± 0.2 nmol/kg and 86 ± 5 ng GH/ml plasma respectively. The AUC values obtained from the GH release showed a similar pattern as the C_{max} values (data not shown). T_{max} for the GH response was obtained after approximately 10 min (10 ± 2 min) and the GH plasma level returned to the basal level within 120 min.

**Specificity**

To study the specificity of NN703 in swine we evaluated plasma levels of cortisol after dosing with three doses: ED_{50}, 10 × ED_{50} and 100 × ED_{50} for GH release. This was compared with hexarelin and MK677 with almost the same relative doses. No signs of adverse effects were observed with any of the doses tested.

In Fig. 5 data are shown for cortisol release. All three doses gave rise to a slight increase (P = 0.48, one-way ANOVA) of approximately 50% compared with basal levels, however, no dose-dependency was observed.

Hexarelin induced a 250% stimulation of the cortisol release when stimulated with a dose only ten times higher than the ED_{50} value for GH release (2.0 ± 0.2, mean ± S.E.M., n = 6) and a nearly 400% stimulation with a dose of 100 times the ED_{50} value. The stimulation of cortisol by hexarelin was in a dose-dependent manner. Also MK677 stimulated cortisol release in a dose-dependent manner; 220% and 350% increase compared with basal levels with doses of 22 and 225 times higher than the ED_{50} value for GH release (46 ± 6, mean ± S.E.M., n = 6).

**Pharmacokinetic and pharmacodynamic studies in dogs**

The pharmacokinetic and pharmacodynamic profiles of NN703 were examined in beagle dogs. One i.v. dose (1 μmol/kg) and three ascending single oral doses (1, 5 and 20 μmol/kg) were administered to four dogs. No
adverse effects were observed in the dogs at any of the tested dose levels.

The $C_{\text{max}}$ and AUC of NN703 increased in a dose-proportional manner (Table 3). The $T_{\text{max}}$ of the two highest doses were not significantly different, although all four dogs had shorter $T_{\text{max}}$ after 20 $\mu$mol/kg compared with 5 $\mu$mol/kg. The oral bioavailability ($f$) was 30–35%.

From the i.v. administration (1 $\mu$mol/kg) the plasma elimination half-life was determined to be 4.1 ± 0.4 h (mean ± S.E.M.), the mean systemic clearance 1.35 ± 0.15 l/h per kg (mean ± S.E.M.) and the mean volume of distribution during the elimination phase 8.23 ± 1.62 l/kg (mean ± S.E.M.).

The stimulation of GH release after oral administration of NN703 to dogs was simultaneously investigated. The GH release after oral administration of NN703 increased in a dose-proportional manner (Table 4). Pronounced inter-individual variation in the level of GH release was observed. At the lowest dose two out of four dogs responded to more than four times the basal level. After the highest dose, $T_{\text{max}}$ was significantly shorter compared with the lowest dose (Table 4).

### Body weight gain in rats

To study the long-term effect of NN703 we examined the body weight in rats after 14 days of oral administration. NN703 was dosed once daily with 100 $\mu$mol/kg and the body weight was determined every day (Fig. 6). As shown in Fig. 6, NN703 induced a significant body weight gain as compared with the vehicle-treated group. The effect of the GHS was already present after the first oral dose. The body weight gain continued during the entire study period with no apparent signs of attenuated growth. At termination of the study the accumulated body weight gains were 27.3 ± 2.7 g and 5.6 ± 1.8 g in the NN703- and vehicle-treated groups respectively ($P < 0.001$). The effect was sustained over the entire period and there were no signs of attenuated growth. However, provocative i.v. stimulations with NN703 (3 $\mu$mol/kg) 24 h after termination of the study showed that the plasma GH response was significantly reduced. The areas under the plasma GH AUC were calculated by use of the trapezoidal rule. The AUC was reduced from 2873 ± 483 min/µg per l in the vehicle-treated group to 1117 ± 181 min/µg per l in the NN703-treated group (means ± S.E.M., $P < 0.01$).

IGF-I was measured in zero samples obtained 24 h after termination of the study and showed no differences in content (vehicle-treated 658 ± 9 ng IGF-I/ml and NN703-treated 670 ± 19 ng IGF-I/ml (means ± S.E.M., $P > 0.05$)).

### Discussion

We here describe a specific and orally active GH secretagogue, NN703, derived from ipamorelin. NN703 demonstrates similar in vitro potency and efficacy as ipamorelin and GHRP-6 in releasing GH from rat pituitary cells. The pharmacological profile of NN703 was equal to that of GHRP-6 and ipamorelin (15). This was described by using known antagonists to the GHRPs, [D-Lys3]-GHRP-6 (20), L-692,400 (16) and [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P (16), which inhibited ipamorelin-, GHRP-6- and NN703-mediated responses, as well as the GHRH antagonist [N-acetyl-Tyr1,D-Arg2]-hGHRH (1–29)NH$_2$ (21) which had no effect on the response of the tested compounds.

### Table 3 Pharmacokinetic parameters after oral administration of NN703 to four beagle dogs (means ± S.E.M.).

<table>
<thead>
<tr>
<th>Dose ($\mu$mol/kg)</th>
<th>$C_{\text{max}}$ (nmol/l)</th>
<th>$T_{\text{max}}$ (h)</th>
<th>AUC (0-tn) (nmol/h per l)</th>
<th>$f$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1548 ± 379</td>
<td>0.9 ± 0.2</td>
<td>4024 ± 569</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>5</td>
<td>268 ± 58</td>
<td>2.3 ± 0.6</td>
<td>988 ± 154</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>1§</td>
<td>57</td>
<td>1.5</td>
<td>125</td>
<td>35</td>
</tr>
</tbody>
</table>

§ Pharmacokinetic parameters could only be calculated in one of four dogs at the lowest dose level, due to plasma concentrations of NN703 below or about LOQ.

### Table 4 Pharmacodynamic parameters after oral administration of NN703 to four beagle dogs (means ± S.E.M.). Basal GH levels are not subtracted.

<table>
<thead>
<tr>
<th>Dose ($\mu$mol/kg)</th>
<th>$C_{\text{max}}$ (ng/ml)</th>
<th>Basal GH (ng/ml)</th>
<th>$T_{\text{max}}$ (h)</th>
<th>AUC (0–23 h) (ng/h per ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>49.5 ± 17.8</td>
<td>1.7 ± 0.3</td>
<td>1.8 ± 0.7</td>
<td>98 ± 29</td>
</tr>
<tr>
<td>5</td>
<td>12.2 ± 5.0</td>
<td>2.3 ± 0.7</td>
<td>2.6 ± 0.6</td>
<td>27 ± 8*</td>
</tr>
<tr>
<td>1</td>
<td>3.9 ± 1.4*</td>
<td>1.8 ± 0.5</td>
<td>3.8 ± 0.3*</td>
<td>14 ± 4*</td>
</tr>
</tbody>
</table>

* $P < 0.05$ vs the highest dose.
GHRP-6 is particularly interesting. Figure 6 shows the effect of NN703 (100 μmol/kg) and vehicle on body weight gain in 5-month-old female rats. NN703 was administered orally once daily for 14 days. The results are expressed as means ± S.E.M. (n = 16 rats per group).

Binding studies using the human GHS-R 1A, expressed stably in BHK cells and transiently in COS-7 cells, and 35S-MK677 demonstrated the expected response of GHRP-6, hexarelin, and MK677. NN703 was also able to displace the binding of the radioligand. However, the calculated Kᵦ values of the tested GHSs on both BHK and COS-7 cell membranes showed that NN703 and ipamorelin displaced 35S-MK677 with significantly lower affinity than MK677, GHRP-6, GHRP-2, and hexarelin.

Table 1 summarises the in vitro data from rat pituitary cells, COS-7 and BHK cells for GHRP-2, GHRP-6, hexarelin, ipamorelin, MK677, and NN703. It is interesting to note that in the pituitary-based assay, MK677 is slightly more potent than the other five GHSs (EC₅₀ 0.4 nmol/l vs 1.3–2.7 nmol/l), while in COS-7 and BHK cells transfected with GHS-R 1A, the inhibition of 35S-MK677 binding with the six GHSs shows that ipamorelin and NN703 displace 35S-MK677 with an affinity 300- to 800-fold less than MK677. Whether this discrepancy in rank of potencies is due to the use of different binding epitopes at the GHS-R 1A or the possible existence of alternative receptors is unclear. Since we have previously shown that ipamorelin has the same in vivo potency in swine (15) and in humans (22) as GHRP-6, the dramatic decrease in affinity of ipamorelin in the binding assays compared with GHRP-6 is particularly interesting.

In the functional based IP₃ assay using BHK cells transfected with human GHS-R 1A, we see a similar response of GHRP-6 and MK677 and a significantly (P < 0.05) less potent response with NN703 and ipamorelin.

In the light of recent publications by Muccioli et al. (23) and Ong et al. (24), where an alternative GHS receptor with high affinity for hexarelin but low affinity for MK677 has been described, it is possible to speculate whether the discrepancies between data using the cloned receptor and the rat pituitary cell assay or data from the cloned receptor and in vivo data may be explained by the involvement of such an alternative receptor. However, since no other GHS receptors have yet been characterised these speculations remain unsolved.

The high potency and specificity of ipamorelin in several species, including human (22), and the fact that NN703 discloses the same in vitro profile as ipamorelin, encouraged us to further characterise NN703 with respect to in vivo potency, specificity, and oral bioavailability.

We have previously described swine to be a suitable animal model because of its close resemblance to humans with respect to several physiological aspects (15). The acute effect of NN703, hexarelin, and MK677 on GH release was therefore evaluated in conscious swine. The GHSs were given as an i.v. bolus injection and the plasma GH levels were monitored for 3 h. The studies were designed as open non-balanced dose-escalation studies. To eliminate the risk of introducing carry-over effects, the intervals between doses were chosen to be 72 h, much longer than the plasma half-life of the tested compounds.

In this model, NN703, hexarelin, and MK677 all gave rise to an immediate GH release in a dose-dependent manner, with ED₅₀ values of 155 ± 23 nmol/kg, 2.0 ± 0.2 nmol/kg, and 46 ± 6 nmol/kg respectively. The efficacy of NN703 was similar to hexarelin and to the efficacies previously reported (15) for GHRP-2, GHRP-6, and ipamorelin (P > 0.05). MK677 had a significantly higher E₅₀ (P = 0.02 and P = 0.003 for NN703 and hexarelin respectively).

Hexarelin showed the same magnitude of potency in swine as previously reported for GHRP-2, GHRP-6, and ipamorelin (14, 15), but it is interesting to note that hexarelin is 25- and 75-fold more potent in swine than MK677 and NN703 respectively. This is not in accordance with the various in vitro assays discussed above. Whether the high potency of the peptides (GHRP-2, GHRP-6, hexarelin, and ipamorelin) versus the peptidomimetics (NN703 and MK677) is caused by the presence of lysine in the C-terminal of the peptides, which has previously been reported to be of importance in in vivo studies (25), or that it is simply caused by different binding epitopes of the GHSs in the different assays, needs further exploration.

The low molecular weight and low hydrophilicity of NN703 relative to ipamorelin and the proven GH release in swine encouraged us to evaluate the potential of NN703 to be administered via the oral route. A pharmacokinetic and pharmacodynamic study in beagle dogs was therefore initiated. NN703 was administered orally in three ascending single doses and one i.v. dose. Table 3 shows Cₘₐₓ, Tₘₐₓ, AUC, and the oral bioavailability (f) after oral administration. A clear dose-dependency on Cₘₐₓ and AUC are observed, in contrast to the oral bioavailability which ranged between 30 and 35% independent of the dose. Also a
clear dose-dependency on GH release was observed after oral administration of NN703 to dogs.

Tmax is about 10 min, which is also in agreement with other GHSs in different species, including man (26–29).

The specificity of GHSs has been addressed in various animals in a number of studies and reviews, (17, 30–32, 36). Especially, the adreno-corticotroph axis and prolactin (PRL) secretion have been addressed. In a study in swine (15) the secretion of all pituitary hormones after stimulation with several GHSs was evaluated. We only observed effects on the adreno-corticotroph axis. A stimulation of cortisol was always preceded by an elevation of adrenocorticotropicin (ACTH). It was also concluded that the young swine, as used in this study, was a poor model of PRL secretion, since none of the tested secretagogues released any PRL, although we know from humans that at least hexarelin and GHRP-2 give rise to PRL release (30). However, one may speculate that since, in a human study with ipamorelin, we only observed PRL release with very high doses of ipamorelin (22), we might expect to find the same pattern for NN703, considering the similar receptor pharmacology.

With respect to the hypothalamic-pituitary-adrenal axis, as mentioned above, swine were very good in predicting the cortisol release found in the human after stimulation with ipamorelin (22). Thus, we have chosen only to evaluate cortisol release in swine after i.v. administration and compare it with hexarelin and MK677, which has previously been described to release cortisol in humans (33, 34). The cortisol release is most likely mediated by ACTH release (15), which is in agreement with earlier findings in swine, where the effect on cortisol is abolished after disconnection of the hypothalamic stalk (26).

As mentioned above, cortisol release in swine was studied after administration of ipamorelin, GHRH, GHRP-2 and GHRP-6 (15). In this study only GHRP-2 gave rise to a significant increase in cortisol levels, whereas ipamorelin and GHRP-6 released cortisol at the same magnitude as GHRH (15). Here we compared the release of cortisol after administration of NN703, hexarelin and MK677. The results show that NN703, administered in doses up to 65 times the ED30 value for GH release, gives rise to an increase of cortisol of approximately 50% compared with basal values. Although this is a significant increase it was not dose-dependent and, more importantly, it was not significantly different (P > 0.05) from that which has previously been found after administration of GHRH (15), which we consider as the optimal physiological reference compound for such studies. In contrast, hexarelin increased cortisol levels by 400% compared with basal values; even at a dose only ten times the ED30 value for GH release we see a stimulation of 250%. The same magnitude in cortisol release has been observed in humans (34). MK677 also stimulates cortisol release in a dose-dependent manner, with a 225% increase in cortisol compared with basal levels, in a dose 22 times higher than the GH ED30 value and 350% increase in cortisol in a dose 250 times the ED30 value for the GH release.

To study the long-term effect of NN703 we examined the body weight in rats after once daily oral administration for 14 days. We have previously observed that the rat responds to GHSs with a much lower potency than many other species (PB Johansen, unpublished results), but nevertheless it is possible to obtain a significant acute response after an i.v. bolus injection of NN703. With this in mind, NN703 was dosed once daily with 100μmol/kg and the body weight was determined every day (Fig. 6). The results showed that the rats which were dosed with NN703 increased their body weight significantly compared with a control group which received only saline. At the termination of the study provocative i.v. stimulation was performed with NN703 and the GH response of NN703 was reduced to 40% in the treated group compared with the vehicle-treated group. The effect on the body weight gain, however, persisted during the entire treatment period with no apparent signs of attenuated growth, despite a reduced GH response to a provocative i.v. stimulation. It is surprising that no rise in IGF-I levels was observed. This is in accordance with an earlier study (35), where longitudinal bone growth was stimulated in rats using ipamorelin without any increase in IGF-I levels 24 h after terminating the study. In other published rat studies (36, 37), IGF-I values have not been addressed, although significant effects of GHS have been demonstrated. One might speculate whether the IGF-I parameter is a good marker for GHS effects.

In conclusion, we have described a new GHSs, NN703, derived from ipamorelin with a similar in vitro profile as ipamorelin, but with a different receptor pharmacology to that of GHRP-6 and MK677. NN703 demonstrates good in vivo potency and efficacy in swine, no dose-dependent increase of cortisol and good oral bioavailability in dogs. Additionally, we have shown that the body weight gain is significantly increased in rats after an oral administration of NN703 once daily for 2 weeks. Based on these data, NN703 was chosen as a clinical candidate and is currently in clinical trials.

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