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Synthesis and biological evaluation of an orally active ghrelin agonist that stimulates food consumption and adiposity in rats

Charles W. Lugar, Michael P. Clay, Terry D. Lindstrom, Andrea L. Woodson, David Smiley, Mark L. Heiman and Jeffrey A. Dodge*

Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285, USA

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Abstract—2-(2-Amino-2-methyl-propionylamino)-5-phenyl-pentanoic acid {1-[1-(4-methoxy-phenyl)-1-methyl-2-oxo-2-pyrrolidin-1-yl-ethyl]-1*H*-imidazol-4-yl}-amide (LY444711, **6**) is an orally active ghrelin agonist that binds with high affinity to and is a potent activator of the growth hormone secretagogue receptor 1a (GHS-R1a) receptor. In rat models of feeding behavior and pharmacology, **6** creates a positive energy balance and induces adiposity by stimulating food consumption and sparing fat utilization. As an orally active ghrelin agonist, **6** represents a new pharmacological tool to investigate the orexigenic role of ghrelin in regulating energy homeostasis.

Ghrelin (**1**) is a 28 amino acid peptide produced predominantly by the stomach and intestines that has recently been identified as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R1a).[1] It is uniquely characterized by an *n*-octanoyl moiety on the Ser3 residue that is derived from a post-translation modification and is essential for biological activity. Ghrelin has been shown to induce adiposity in rodents by increasing food intake and decreasing the utilization of fat tissue.[2] In humans, ghrelin stimulates appetite and induces food intake.[3] Other physiological effects of this hormone include the stimulation of GH secretion from the pituitary in rats and humans.[4,5] Recent studies suggests that the adipogenic properties of ghrelin may be independent from its ability to stimulate GH secretion from the pituitary.[2] Ghrelin likely induces its effects on feeding and energy balance by binding the GHS-R1a on the NPY and Agouti-related protein (AGRP) neurons of the arcuate nucleus of the hypothalamus[6] and induces the synthesis[7] and release of these anabolic neuropeptides. While extensive efforts have been directed toward optimizing molecules that stimulate pituitary

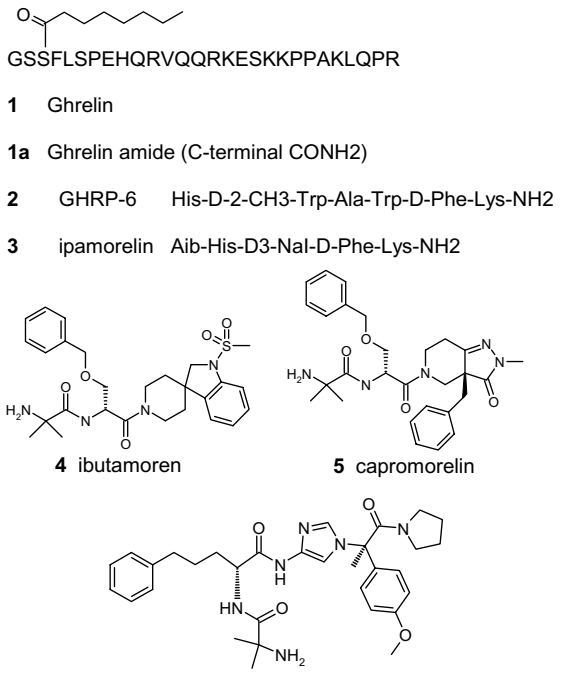

Figure 1. Structures of ghrelin (human) analogs and growth hormone secretagogues.

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* Corresponding author. Tel.: +1 3172768252; fax: +1 3172772035; e-mail: jdodge@lilly.com

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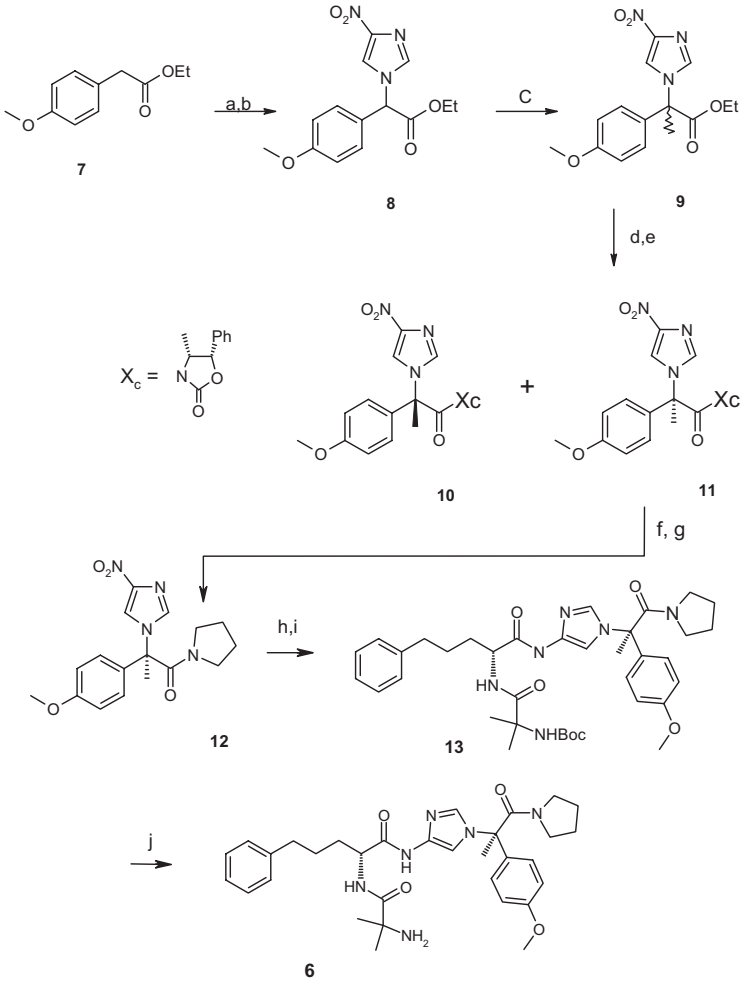
GH release, such as growth hormone releasing peptide GHRP-6 (**2**), ipamorelin (**3**), ibutamoren (**4**), capromorelin (**5**), and others,[8] the effects of GHSs on appetite regulation and energy homeostasis have remained considerably less characterized. In fact, despite the fact that orally active small molecule GHS ligands are well documented, only peptide ligands with poor oral activity, such as ghrelin, **2**, and **3**, have been prospectively characterized for their adipogenic effects to date.[2,9] Herein, we describe the synthesis and the pharmacology of **6**, a ghrelin agonist that represents an orally active pharmacological tool to investigate the orexigenic and anabolic role of ghrelin in regulating energy homeostasis (Fig. 1).

A convergent synthesis of **6** is outlined in Scheme 1. Bromination of ethyl 4-methoxyphenyl acetic acid (**7**) with *N*-bromosuccinamide followed by regioselective N-alkylation[10] of 4-nitroimidazole yielded **8**. Treatment of **8** with sodium bis(trimethylsilyl)amide followed by methyl iodide gave **9**, which was subsequently saponified with lithium hydroxide to give the corresponding carboxylic acid. This racemic acid was resolved by formation of diastereomers **10** and **11** by reaction of the acid chloride of **9** with the lithium anion of (4*R*,5*S*)-(+)-4-methyl-5-phenyl-2-oxazolidinone. The resulting 1:1 mixture of diastereomers, **10** and **11**, were readily separated by silica gel chromatography. The absolute stereochemistry of the chiral quaternary center was assigned as *S* for **10** and *R* for **11** based on the X-ray crystal structure obtained for **10**. Conversion to **12** was accomplished by removal of the oxazolidinone and formation of the corresponding pyrrolidine amide. Reduction of **12** with hydrogen in the presence of palladium yielded a relatively unstable 4-aminoimidazole, which was not isolated but coupled directly with (*R*)-2-(2-*tert*-butoxycarbonylamino-2-methyl-propionylamino)-5-phenyl-pentanoic acid[11] to yield **13**. Deprotection of **13** using HCl yielded **6**.

Compound **6** was evaluated for its ability to bind to the human ghrelin receptor (GHS-R1a) and to act as a functional agonist. In a competitive binding assay,[1] **6** displaced I^{125}-ghrelin with an affinity constant (K_i) of 10.6 ± 3.2 nM ($n = 23$). In AV-12 cells transfected with the GHS-R1a receptor,[12] **6** is a potent agonist with an EC_{50} of 1.2 ± 0.2 nM ($n = 5$) in stimulating calcium



Scheme 1. Reagents and conditions: (a) *N*-bromosuccinamide, AIBN; (b) 4-nitroimidazole, K_2CO_3; 61% two steps; (c) sodium bis(trimethylsilyl)amide, methyl iodide, 92%; (d) LiOH; (e) oxalyl chloride, (4*R*,5*S*)-(+)-4-methyl-5-phenyl-2-oxazolidinone (Xc), *n*-BuLi gave **10** (41%), and **11** (41%); (f) LiOH; (g) oxalyl chloride, pyrrolidine; (h) H_2, 5% Pd/C; (i) *R*-2-(2-*tert*-butoxycarbonylamino-2-methyl-propionylamino)-5-phenyl-pentanoic acid, DCC, HOBt; (j) step 1 HCl; step 2 $NaHCO_3$.

mobilization as determined by fluorescence light induced polarization.

In order to assess the viability of **6** as an orally active chemical tool for studying ghrelin pharmacology, we initially evaluated the stability of **6** versus that of ghrelin amide **1a** in rat plasma at 37 °C. We used **1a** since C-terminal modifications of peptides are generally known to decrease their susceptibility to enzymatic degradation. We found that **1a** undergoes rapid decomposition when incubated in rat plasma. After a 1 h incubation period, only 61% of the parent peptide remained and after 6 h 18% remained. In contrast, **6** remained unchanged after 6 h when exposed to rat plasma under conditions identical to that of ghrelin amide. The oral bioavailability of **6** was subsequently evaluated in rats and dogs. Oral administration of a 30 mg/kg dose of **6** to male F344 rats ($n = 3$ rats/time point) resulted in a maximal concentration (C_{max}) of 211 ± 76 ng/mL in 2 h. The area under the curve AUC (0-inf.) was 633 ng h/mL with a half-life of 1 h. The bioavailability of **6** was determined to be 10% in rats and 37% in beagle dogs.

The pharmacological profile of **6** as a ghrelin agonist was assessed by measuring carbohydrate and fat utilization in rodents by indirect calorimetry using respiratory quotient (RQ) over a 24 h period (Fig. 2).[13–15] A single oral administration of **6** induced an increase of the RQ in a dose-dependent manner. This increase is especially evident during the dark photoperiod when the animals are consuming the majority of their daily fuel intake. As shown in Table 1, significant decreases in lipid oxidation at the 10 mg/kg (27.9 ± 2.44 kcal/kg/d, $p = 0.04$) and 30 mg/kg (27.9 ± 6.32 kcal/kg/d, $p = 0.04$) groups are observed when compared to vehicle-treated animals (61.66 ± 13.62 kcal/kg/d). These doses significantly increased carbohydrate utilization (115 ± 5.49 kcal/kg/d, $p = 0.02$ and 124 ± 6.18 kcal/kg/d, $p = 0.008$, respectively) compared to vehicle-treated controls (81.7 ± 11.8 kcal/kg/d). The observed increase in the use of carbohydrates of the food consumed to meet energy requirements results in the sparing of glycogen synthesis and fat stores, data which is consistent with the increase in body fat observed after two weeks of dosing (vide infra). Food consumption tended to increase in proportion to the amount of compound administered, with the food intake at 30 mg/kg exceeding that of vehicle-treated controls by 130% ($p = 0.06$).

Table 1. Daily fuel balance (kcal/kg/d) for male Long–Evans rats fed a caloric dense diet[a]

Caloric source	Dose, mg/kg	Caloric intake	Caloric utilization
Protein	Vehicle	42.7 ± 5.2	33.0 ± 1.0
	3	51.1 ± 1.5	32.5 ± 0.2
	10	52.2 ± 1.9	32.9 ± 0.7
	30	55.7 ± 2.2	32.9 ± 0.7
Fat	Vehicle	91.3 ± 11.1	61.7 ± 13.6
	3	109.3 ± 3.2	32.3 ± 3.6
	10	111.6 ± 4.1	27.9 ± 2.4*
	30	119.2 ± 4.8	27.9 ± 6.3*
Carbohydrate	Vehicle	94.3 ± 12.8	81.7 ± 11.8
	3	112.8 ± 3.4	109.0 ± 3.9
	10	115.2 ± 4.2	115.1 ± 5.5*
	30	123.0 ± 5.0	124.4 ± 6.2*

* $p < 0.05$.

[a] Animals were treated ($n = 4$/group) with LY444711 or vehicle by oral gavage.

Chronic treatment of LY444711 (po, 10 mg/kg/d) for 2 weeks in Long–Evans rats ($n = 5$/group) significantly increased body weight gain by day 2 of treatment ($p = 0.037$) and by day 14, animals receiving **6** had gained 23% more body weight than vehicle-treated control animals ($p = 0.049$) (Fig. 3). Cumulative food consumption of treated animals was significantly greater than that of vehicle-treated control rats beginning on day 4 ($p = 0.018$) (Fig. 4).

Analysis of body composition before and following 2 weeks of treatment with **6** was performed by dual-energy X-ray absorptiometry (DEXA) to determine the relative

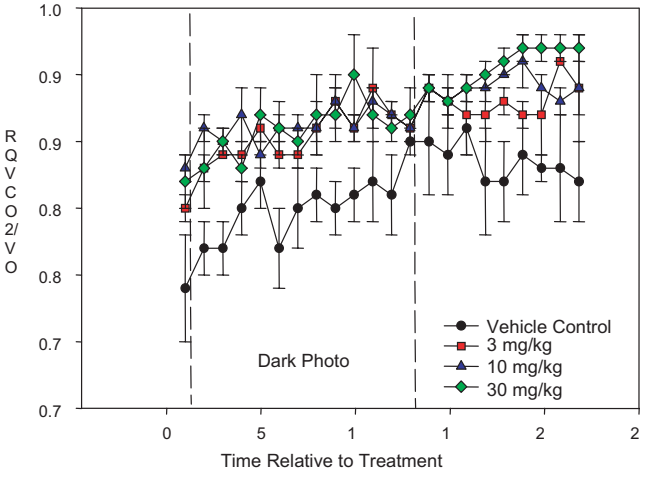


Figure 2. Respiratory quotient (RQ) over a 24 h period after oral administration of LY444711. RQ (y-axis) is defined as the volume of carbon monoxide produced (V_{CO_2})/volume of oxygen consumed (V_{O_2}).

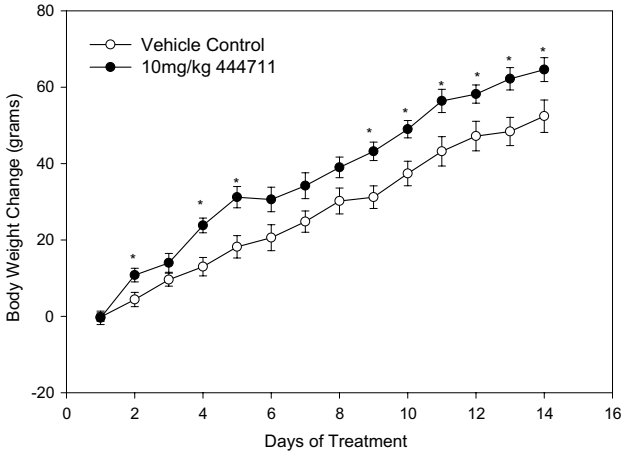


Figure 3. Oral administration of LY444711 increases body weight in rats.

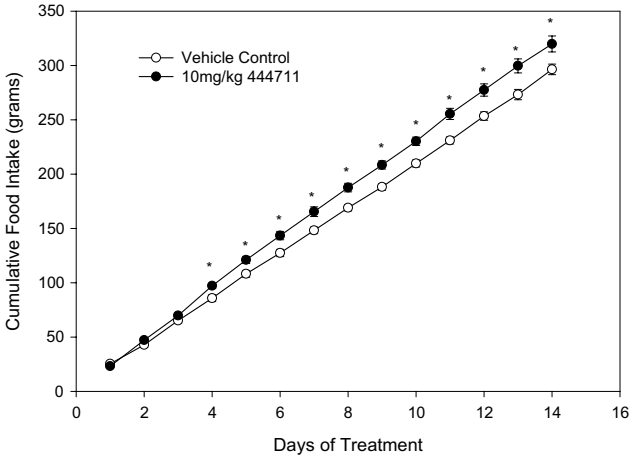


Figure 4. Oral administration of LY444711 increases cumulative food intake in male rats.

contributions of changes in fat and lean mass to the observed increase in body weight gain.[16] Administration of **6** resulted in an increase of fat mass (74.7 g ± 6.6), which was significantly greater than the gain of fat mass observed in vehicle-treated control animals (47.2 g ± 7.6, $p = 0.026$). No change in lean mass (22.7 g ± 5.7 and 26.7 g ± 4.9, respectively, $p = 0.61$) or bone area was noted (19.0 cm^2 ± 2.5 and 14.7 cm^2 ± 2.0, respectively, $p = 0.2$). Serum IGF-1 levels did not differ between groups at the end of the experimental period ($p = 0.59$) indicating that the observed changes in body composition and energy balance induced with **6** may be independent of its ability to stimulate pituitary GH release. However, **6** elevated plasma GH and IGF-1 in beagle dogs after a single oral dose of 1 mg/kg.[17]

LY444711 (**6**) is an orally active ghrelin agonist. This compound binds with high affinity to the GHS-R1a receptor and stimulates calcium mobilization in AV-12 cells transfected with the human ghrelin receptor. This compound is significantly more stable in rat plasma than ghrelin amide **1a** and has good plasma exposure after oral administration to rats. Like ghrelin, **6** creates a positive energy balance when administered acutely to rodents. When administered chronically, **6** induces adiposity by stimulating food consumption and sparing fat utilization. As an orally active ghrelin agonist with a prolonged half-life relative to ghrelin, **6** represents a new pharmacological tool to investigate the orexigenic role of ghrelin in regulating energy homeostasis.

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