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Design and biological activities of L-163,191 (MK-0677): A potent, orally active growth hormone secretagogue

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ABSTRACT **A potent, orally active growth hormone (GH) secretagogue L-163,191 belonging to a recently synthesized structural class has been characterized. L-163,191 releases GH from rat pituitary cells in culture with EC_{50} = 1.3 ± 0.09 nM and is mechanistically indistinguishable from the GH-releasing peptide GHRP-6 and the prototypical nonpeptide GH secretagogue L-692,429 but clearly distinguishable from the natural GH secretagogue, GH-releasing hormone. L-163,191 elevates GH in dogs after oral doses as low as 0.125 mg/kg and was shown to be specific in its release of GH without significant effect on plasma levels of aldosterone, luteinizing hormone, thyroxine, and prolactin after oral administration of 1 mg/kg. Only modest increases in cortisol were observed. Based on these properties, L-163,191 has been selected for clinical studies.**

Interest in growth hormone (GH) secretagogues has intensified during the past several years based on promising, ever-widening investigational applications of recombinant GH in animals and in humans. However, the utility of recombinant GH is limited by the need to administer it by injection. In addition, side effects, including carpal tunnel syndrome, have been reported during treatment (1). It has been suggested that secretagogues may offer an advantage by stimulating a more physiologically relevant, pulsatile release of GH (2). Such secretagogues include the GH-releasing peptide (GHRP)-6 (His-D-Trp-Ala-D-Phe-Lys-NH_2) (3, 4) and the recently announced nonpeptide L-692,429 (5).

The clinical efficacy and specificity of the GHRPs in elevating GH have been established (6) and more recently GH release in humans has been reported by using the GHRP-6 analog hexarelin (7, 8). Furthermore, the nonpeptide L-692,429 (MK-0751) (Fig. 1) has been shown to elevate GH in young male volunteers (9) and in healthy older subjects (10) with only small transient increases in cortisol and prolactin. Thus, the effectiveness of the GHRP-6 mechanism has been established in the clinic with peptides as well as a nonpeptide. Unfortunately, these secretagogues have limited oral bioavailability.

The design of an additional class of orally active secretagogues originated in a project to derivatize "privileged structures" as a strategy to discover leads for G-protein receptors. It has been appreciated for many years that certain molecular units have the capability to interact with diverse receptors, a phenomenon for which Evans *et al.* (11) coined the phrase privileged structures. For example, structural templates such as benzodiazepines and 1,1-diphenylmethanes were known to be recurring structural features of some central nervous system drugs (12, 13). A benzodiazepine core is present in the natural product cholecystokinin (CCK) antagonist asperlicin, and Evans *et al.* (14) derivatized that part structure in their innovative development of potent, orally active nonpeptide CCK antagonists. They further suggested that judicious modification of privileged structures could be a useful approach to develop receptor agonists or antagonists.

In our own case, testing compounds from other receptor projects uncovered modest activity in compound **1**, which released GH in our pituitary cell assay with EC_{50} = 300 nM. The spiropiperidine part structure of this compound is a privileged structure in the terminology of Evans *et al.* (11), since it is also found, for example, in oxytocin (15) and sigma receptor ligands (16). We derivatized the spiropiperidine core of **1** and from this approach lead compound **2** was discovered. It is remarkably potent, with ED_{50} = 50 nM, even as a mixture of four diastereomers, although, unfortunately, it also was poorly active to release GH in dogs after oral administration.

Modifications of compound **2** to enhance potency and improve *in vivo* properties were undertaken with the synthesis of numerous analogs to optimize each structural component of compound **2.** Fig. 2 highlights the potencies of some of these analogs, which culminated in the selection of compound **13** for detailed studies. Its superior oral potency and duration of action led to safety assessment studies and to ongoing clinical trials under the designations L-163,191 and, as the mesylate salt, MK-0677.

MATERIALS AND METHODS

Chemistry. The compounds shown in Fig. 2 were prepared by coupling *N-tert*-butoxycarbonyl (Boc) D-amino acids to spiropiperidines by using 1-hydroxybenzotriazole and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride followed by removal of the Boc protecting group under standard peptide synthesis conditions. Analogs bearing the aminoisobutyric acid side chain were synthesized by carrying out a second peptide-type coupling reaction with *N-t*-Boc aminoisobutyric acid again followed by Boc removal. Compounds **5** and **6** were made following the methods of Schoen *et al.* (17) and Ok *et al.* (18). The required parent 2,3-dihydro(1*H*-indane-1,4′-piperidine) is a known compound (16) as is *N*′-methyl spiro(indoline-3,4′-piperidine) (19), which was acylated and demethylated to provide intermediates for the synthesis of compounds **11**, **12**, and **13.** 3-Oxospiro(indane-1,4′-piperidine) was prepared from the Boc protected spiro(1*H*-indene-1,4′-piperidine) (16) by hydroboration with 9-borabicyclo-[3.3.1]nonane followed by oxidation with pyridinium chlorochromate and deprotection of the Boc group with trifluoroacetic acid. Compound **10** was synthesized from **9** by carrying out a sodium borohydride reduction in methanol. *N-t*-Boc D-tryptophan and *N-t*-Boc *O*-benzyl-D-serine were purchased from commercial sources.

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Abbreviations: GH, growth hormone; GHRP, GH-releasing peptide; Boc, butoxycarbonyl; GHRH, GH-releasing hormone.

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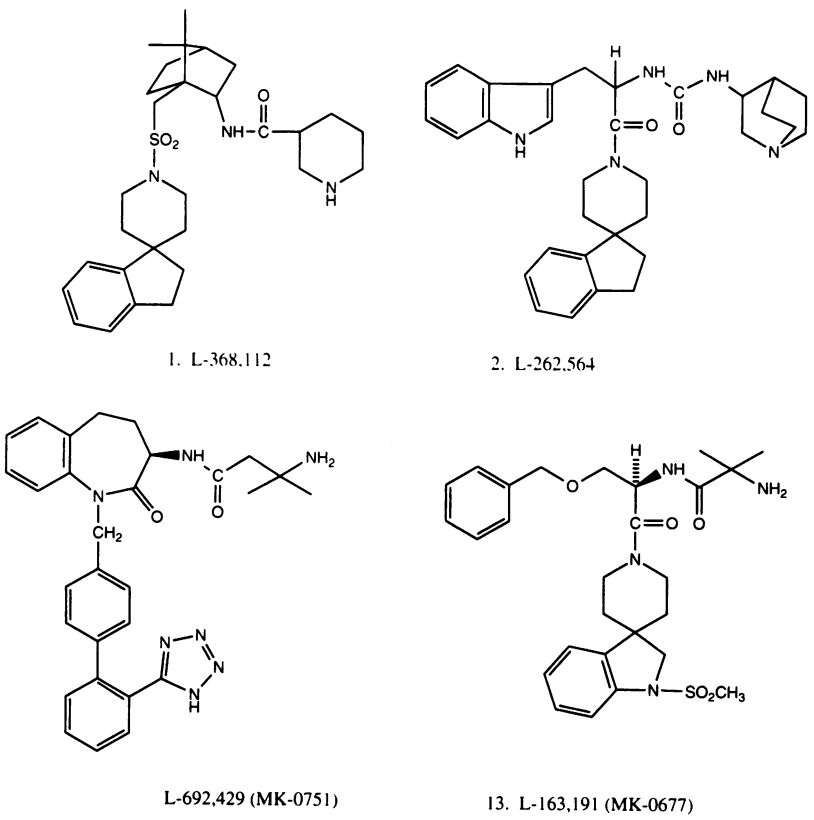


FIG. 1. GH secretagogue structures.

The compounds shown in Fig. 2 were isolated usually as their hydrochloric acid or trifluoroacetic acid salts. Initial *in vivo* evaluations were carried out with hydrochloric acid salts. All the compounds reported in Fig. 2 were fully characterized by consistent ^{1}H NMR (400 MHz) and fast atom bombardment mass spectra. *N*-[1(*R*){[1,2-dihydro-1-methanesulfonylspiro-(3*H*-indole-3,4′-piperidin)-1′-yl]carbonyl}-2-(phenylmethoxy)-ethyl]-2-amino-2-methylpropanamide methanesulfonate (**13**; L-163,191, MK-0677) is a white to off-white crystalline powder isolated as a monohydrate that melts at 168°–170°C. Its solubility in distilled water is >100 mg/ml. The pK_a of the compound is 7.8.

Pituitary Cell Culture Assays. In a typical experiment, pituitary glands were aseptically removed from Wistar male rats (150–200 g) and cultures of pituitary cells were prepared according to Cheng *et al.* (20). The cells were treated with various GH secretagogues and assayed for GH secreting activity again as described (20). Intracellular cAMP levels were also measured according to Cheng *et al.* (20).

Calcium Assay in Rat Somatotrophs. After 3 days of culturing, rat pituitary cells were dispersed with trypsin (0.25 mg/ml). The cells were attached to a glass coverslip coated with poly(L-lysine) and somatotrophs were identified by a reverse hemolytic plaque assay (21). The cells on the glass coverslip were then loaded with 1 μM fura-2/AM {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethyl ester} and the internal free Ca^{2+} concentration in each somatotroph was measured with a fluorescence ratio image technique as described (5).

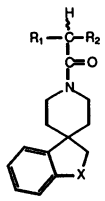
Intravenous and Oral Efficacy Studies in Dogs. For the intravenous study, L-163,191 was given to two or four conscious beagles in doses of 0.01, 0.025, 0.05, and 0.1 mg/kg as indicated in the legend of Fig. 5. The beagles were 1.5–2.5 years old, weighed 8–16 kg, and both males and females were used. They were catheterized via the cephalic vein and the treatments were infused over a 5-min period in a volume of saline equal to 2 ml per kg of body weight. Dogs were bled from the jugular vein at −5, 0 (before the start of infusion), 5, 15, 25, 35, and 45 min. From the blood collected at each sampling, the sera were separated and assayed for GH via RIA.

In the oral study, doses of 0, 0.25, 0.50, and 1.0 mg of L-163,191 per kg were administered to eight dogs (four males and four females) in a randomized, balanced crossover design. Treatments were given at 7-day intervals over 4 weeks. Each treatment was administered via stomach tube in a volume of water equal to 5 ml per kg of body weight. Dogs were bled from the jugular vein −5, 0, 15, 25, 35, 45, 65, 90, and 120 min after dosing. From the blood collected at each sampling, the sera were separated and assayed for GH. In both studies, the dogs were fasted for 15 hr before and during experimentation.

Eight-Hour Hormone Profiles in Dogs. Six male beagles (1.5–2.0 years old) weighing 9.5–12.5 kg were used. The dogs were fasted for 15 hr before and during experimentation. Three dogs were dosed orally with L-163,191 at 1 mg/kg and three dogs were dosed with placebo (water). The drug was administered in a volume of water equal to 5 ml per kg of body weight via stomach tube. Dogs were bled from the jugular vein −10, 0, 15, 30, 60, 90, 120, 240, 360, and 480 min after dosing. From the blood collected at each sampling, the sera were separated and stored at −20°C for determination of GH, cortisol, aldosterone, luteinizing hormone, prolactin, thyroxine, and insulin-like growth factor 1.

Hormone Analysis. All hormone analyses were performed by RIA. The GH assay was performed at the Merck Research Laboratories and others were done at the Veterinary Diagnostic Laboratory (Cornell University, Ithaca, NY). Each assay had been validated for dog sera and/or plasma and their sensitivities and intra- and interassay coefficients of variations were as reported (22).

Statistical Analysis. For statistical analyses, at each time point the geometric mean hormone response was plotted against time. Trapezoidal area under the curve from 0–8 hr after dosing was computed for each hormone. Two sample *t*



Compound	R_1	R_2	X	Stereo	EC_{50}(nM)
2	3-indolylmethyl-	NHC(O)NH- 	CH_2	D, L	50
3	3-indolylmethyl-	$NHC(O)C(CH_3)_2NH_2$	CH_2	L	10,000
4	3-indolylmethyl-	$NHC(O)C(CH_3)_2NH_2$	CH_2	D	14
5	3-indolylmethyl-	$NHC(O)CH_2C(CH_3)_2NH_2$	CH_2	D	76
6	3-indolylmethyl-	$NHC(O)CH_2C(CH_3)_2NHCH_2CH(--OH)CH_3$	CH_2	D	2.6
7	3-indolylmethyl-	$NHC(O)CH_2C(CH_3)_2NHCH_2CH(--OH)CH_2OH$	CH_2	D	16
8	benzyloxymethyl-	$NHC(O)C(CH_3)_2NH_2$	CH_2	D	17
9	3-indolylmethyl-	$NHC(O)C(CH_3)_2NH_2$	C=O	D	1.2
10	3-indolylmethyl-	$NHC(O)C(CH_3)_2NH_2$	CH~OH	D	0.62
11	3-indolylmethyl-	$NHC(O)C(CH_3)_2NH_2$		D	4.2
12	3-indolylmethyl-	$NHC(O)C(CH_3)_2NH_2$	NSO_2CH_3	D	1.8
13	benzyloxymethyl- (L-163,191; MK-0677)	$NHC(O)C(CH_3)_2NH_2$	NSO_2CH_3	D	1.3
14	benzyloxymethyl-	$NHC(O)C(CH_3)_2NH_2$	NSO_2CH_3	L	500

FIG. 2. Intrinsic GH secretory potency in the pituitary cell assay. Stereo designates stereochemistry at the carbon atom bearing the R_1 and R_2 substituents. EC_{50} for half-maximal release of GH in the rat pituitary cell assay normalized for L-692,429 is 60 nM.

tests were used to compare the response to L-163,191 and placebo. Any two means were significantly different ($P < 0.05$) if their error bars did not overlap.

RESULTS AND DISCUSSION

The design breakthrough in this series of GH secretagogues was provided by compound **2.** The fact that it contains tryptophan may well have contributed to its efficacy since, in the genesis of GHRP-6, the key change that converted [Met]-enkephalin amide into a GH secretagogue lead was the introduction of D-tryptophan in the 2 or 3 position of this opiate ligand (23). The marked preference for D stereochemistry in the current compounds (compare compounds **3** and **4**; **13** and **14**), which is identical to that in the benzolactam secretagogue L-692,429 (5), suggests but does not establish possible peptidyl and nonpeptidyl correspondence at the D-tryptophan residue of GHRP-6. Furthermore, it is worth noting that the quinuclidine group in compound **2** was present in an unpublished GH secretagogue screening lead from the Merck sample collection. Thus, all three components of compound **2** were known to us in GH secretagogue leads and their fortunate combination afforded the current lead.

A relationship of the spiro compounds to the benzolactam secretagogues including L-692,429 is apparent in the stereochemistry mentioned above and in their N-terminal amino acid derivatization as illustrated in compounds **4, 5,** and **6.** These same amino acids had afforded highly active benzolactam secretagogues and our use of them and other N-terminal amino acid derivatizations were guided by that earlier research.

Potency was markedly enhanced by derivatizing the indane 3 position of compound **7** with polar groups including the ketone **9** and alcohol **10.** Equivalent activities were obtained by using acylated 1,2-dihydrospiro(3*H*-indole-3,4′-piperidine) substitutions in compounds **11** and **12.** Also D-tryptophan could be replaced with other D-amino acids including *O*-benzyl-D-serine. With these structural flexibilities, it was possible to study *in vivo* a number of potent compounds with differing physical properties, and from these studies compound **13** (L-163,191; MK-0677) emerged as a clinical candidate. L-163,191 stimulated GH release in the rat pituitary cell assay with $EC_{50} = 1.3 \pm 0.09$ nM. Thus, its intrinsic activity is considerably greater than the benzolactam L-692,429 ($EC_{50} = 59.6 \pm 7.3$ nM) and GHRP-6 ($EC_{50} = 10.3 \pm 1.9$ nM), and it approaches that of the GH-releasing hormone (GHRH) in this assay ($EC_{50} = 0.47 \pm 0.09$ nM) (24).

L-163,191 has many properties in common with the hexapeptide GHRP-6. For example, its pituitary cell activity is antagonized by the GHRP-6 antagonist His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH_2 (K.C., unpublished data). Furthermore, no additional increase in GH release was observed when a maximally effective concentration of L-163,191 was combined with a maximally effective concentration of GHRP-6 (K.C., unpublished data). These results suggest that both compounds mediate GH release via the same receptor.

In contrast, when either L-163,191 or the benzolactam L-692,429 was combined with a maximal concentration of GHRH (10 nM) in the pituitary cell assay, the amount of GH released was greater than the sum of the individual increases (Fig. 3). Neither compound affected basal levels of cAMP; however, they potentiated the effect of GHRH on the accu-

mulation of intracellular cAMP. Similar potentiation has been reported for GHRP-6 (20). Although GHRH and L-692,429 are equally effective in inducing maximal GH release in the pituitary cell assay, in humans L-692,429 is more effective in raising GH levels. These results suggest that secretagogues such as L-692,429 elevate GH *in vivo* by mechanisms in addition to a direct action on the pituitary. Potentiation of GHRH in the pituitary could be one such mechanism. In addition, evidence is accumulating that GHRP-6 and L-692,429 act in the hypothalamus to induce the release of GHRH (25). If so, releasing as well as potentiating GHRH would be of obvious advantage with respect to the *in vivo* efficacy of L-692,429 and other mechanistically related GH secretagogues.

The desensitization of pituitary cells to the action of L-163,191, L-692,429, and GHRP-6 provided additional evidence that all three act via the same mechanism. Their stimulatory signals were turned off at similar rates when pituitary cells were continuously exposed to these compounds, although the cells remained responsive to GHRH (10 nM). Thus, rat pituitary cells pretreated with L-163,191 (100 nM), L-692,429 (1 μM), or GHRP-6 (100 nM) for 1 hr failed to respond to any of these three secretagogues in a subsequent 15-min incubation.

Mechanistic studies using rat somatotrophs demonstrated an elevation of intracellular free calcium levels by L-163,191 through the activation of L-type Ca^{2+} channels. All cells exhibited stable, low levels of cytosolic free calcium (50–100 nM) before addition of L-163,191. In 11 of 14 somatotrophs, the addition of 10 nM L-163,191 elicited transient elevations of cytosolic free calcium with peak concentrations from 250 to 700 nM. In two cells, 1 μM nifedipine was added about 30 or 60 sec before addition of 10 nM L-163,191. Neither of these cells showed elevation of cytosolic free calcium (Fig. 4). This is consistent with our earlier findings that GHRP-6 and L-692,429 cause GH release by activation of L-type Ca^{2+} channels (5).

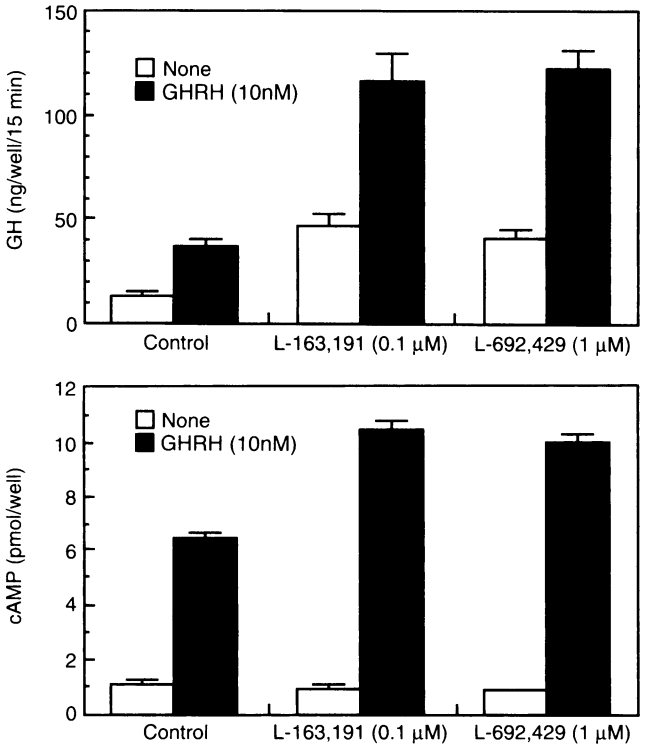


FIG. 3. Synergistic effects between L-163,191 or L-692,429 and GHRH on GH release and cAMP accumulation.

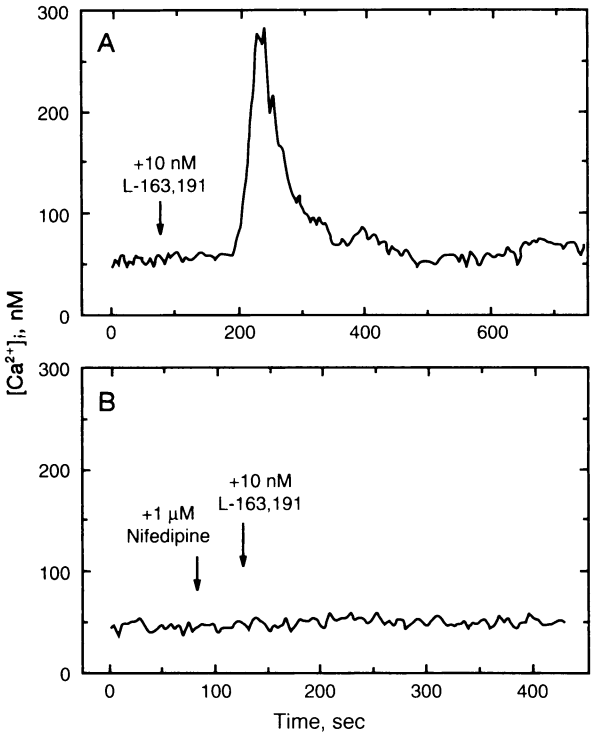


FIG. 4. Effect of nifedipine on induction by L-163,191 of intracellular free Ca^{2+} concentration in rat somatotrophs as determined by fluorescence ratio imaging. Graphic representatives of the internal free Ca^{2+} concentration in response to L-163,191 in the absence (*A*) or presence (*B*) of 1 μM nifedipine.

GHRP-6 (26) and L-692,429 (5) have been reported to mediate GH release via protein kinase C. Similar findings have been obtained with L-163,191 (K.C., unpublished data). Thus, in all aspects examined, the mechanism of action of L-163,191 has been found to be identical to that of earlier described peptide and nonpeptide GH secretagogues. The *in vitro* specificity of L-163,191 was demonstrated in >50 receptor assays in which IC_{50} values were >10 μM. These included opiate, sigma, angiotensin II, bradykinin, serotonin, muscarinic, neurokinin, galanin, vasopressin, benzodiazepine, and endothelin assays.

The intravenous administration of L-692,429 to dogs proved to be a good predictor of its intravenous potency in humans. Therefore, we relied on dog models to evaluate analogs in the present series of compounds. The best of these secretagogues, L-163,191, elevated GH levels intravenously in beagles as low as 0.025 mg/kg (Fig. 5), at which dose the peak GH response

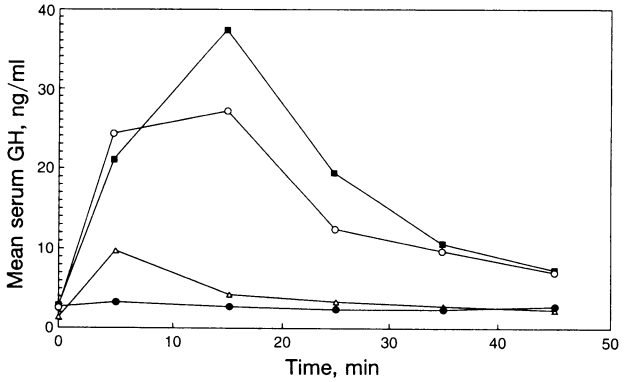


FIG. 5. Mean serum growth hormone in beagles given L-163,191 intravenously. Dose of L-163,191 (mg/kg): ●, 0.01 (n = 4); △, 0.025 (n = 2); ○, 0.05 (n = 2); ■, 0.1 (n = 2).

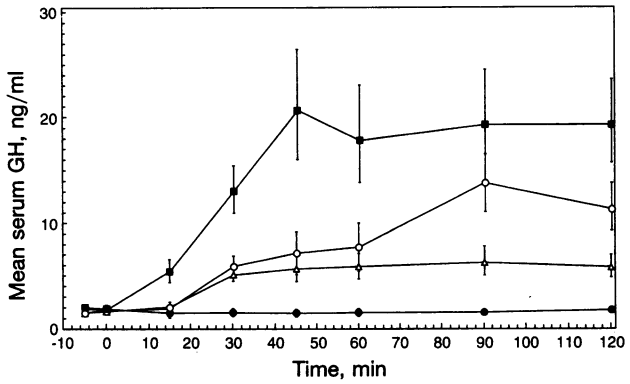


FIG. 6. Geometric mean serum GH concentrations (± within-dog SE) after single oral administration of L-163,191 to eight beagles. Dose of L-163,191 (mg/kg): ●, placebo; △, 0.25; ○, 0.50; ■, 1.0.

and duration of action were comparable to L-692,429 at 0.1 mg/kg (22). Significant increases in GH were observed at oral doses as low as 0.125 mg/kg in 8 of 10 dogs. In a dose range study with eight dogs in a crossover design (Fig. 6), significant increases were seen in GH levels in a dose-dependent fashion with a duration of action that exceeded 2 hr. From a comparison of the plasma concentrations of L-163,191 after oral and intravenous administration to dogs, its oral bioavailability was estimated to be >60% (K.H.L. and S.-H.L.C., unpublished data). In contrast L-692,429 had negligible oral activity in dogs and the oral bioavailability of GHRP-6 in humans has been reported to be <1.0% (27). We ascribe the excellent bioavailability of L-163,191 to its lipophilicity (log P = 3.0) in combination with remarkably good water solubility as its crystalline mesylate salt. Furthermore, its peptidase stability is high since the amino acids it contains, *O*-benzyl-D-serine and α-methylalanine, generate a poor substrate for peptidase cleavage.

Additional studies have demonstrated good dose-dependent oral activity in rats and swine (T.J. and G.H., unpublished data). The selectivity of L-163,191 with respect to cortisol, aldosterone, prolactin, luteinizing hormone, and thyroxine was evaluated orally in dogs (n = 3). At 1.0 mg/kg, GH area under the curve over 8 hr was increased ≈ 9-fold (Fig. 7) and cortisol was increased 2.4-fold, while aldosterone, luteinizing hormone, prolactin, and thyroxine levels were not significantly altered. Serum insulin-like growth factor 1 levels were elevated by 30% at 8 hr. Thus, L-163,191 is a selective GH secretagogue with the exception of modest increases in cortisol. Similar small elevations of cortisol were reported with L-692,429 in dogs (22) and in humans (9, 10). Also, some cortisol increases have been reported with GHRP-6 in humans (28). Thus, despite considerable structural differences, L-163,191 produces, along with these earlier secretagogues, modest elevations in cortisol level. It remains to be determined whether this property is inherent in the GHRP mechanism of action and whether it is clinically significant.

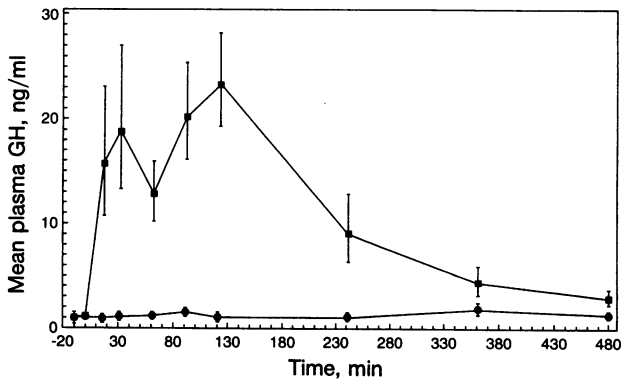


FIG. 7. Mean plasma GH levels after oral administration of L-163,191 (1.0 mg/kg). Dose of L-163,191 (mg/kg): ●, placebo; ■, 1.0. Nonoverlapping error bars imply statistical significance.

In summary, we report here a potent GH secretagogue that also has excellent oral bioavailability. Its discovery now permits expanded animal and clinical studies to explore the potential value of GH secretagogues in medicine.

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