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Preclinical Pharmacology of CP-424,391, an Orally Active Pyrazolinone-Piperidine Growth Hormone Secretagogue

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Growth hormone secretagogues (GHSs) represent attractive therapeutic alternatives to recombinant growth hormone (GH), given their ability to amplify pulsatile hormone secretion in a relatively physiologic manner. CP-424,391 (391) is a novel, orally active pyrazolinone-piperidine GHS. In rat pituitary cell cultures, 391 stimulated GH release with an EC_{50} = 3 n*M*. The addition of 391 to rat pituitary cells activated intracellular calcium signaling but did not elevate intracellular cyclic adenosine monophosphate (cAMP). 391 also modulated the effects of GH-releasing hormone and somatostatin on pituitary cell GH-release and intracellular signaling. In nonpituitary cell lines, the ability of 391 to stimulate intracellular signaling was dependent on the expression of recombinant human GHS receptor. Acute administration of 391 to anesthetized rats or to conscious dogs induced pulsatile release of GH in a dose-dependent manner. Plasma insulin-like growth factor-I (IGF-I) was elevated progressively over a 5-d course of daily oral dosing in dogs. Chronic oral administration of 391 augmented body weight gain in rats and dogs. Thus, the peptidomimetic GHS 391 has potential utility for the treatment of clinical conditions that could benefit from systemic augmentation of GH and IGF-I levels.

Key Words: peptidomimetic growth hormone secretagogue; somatotroph; insulin-like growth factor-1; intracellular signaling; pharmacology; cortisol.

Introduction

The growth hormone (GH)/insulin-like growth factor-1 (IGF-1) endocrine system undergoes an age-related decline in its ability to regulate hormonal secretion. In men between the ages of 20 and 70 yr, pituitary GH production decreases by 14% per decade and GH half-life falls by 6% *(1)*. Circulating IGF-I levels in men and women also decline with age *(2)*. This progressive diminution of circulating GH and IGF-I, which is also known as the somatopause, is most evident in men over the age of 50 and has become associated with a spectrum of physiologic changes that are reminiscent of the syndrome of adult GH deficiency (GHDA). The consequences of GHDA include increased visceral adiposity, reduced lean body mass and muscle-to-fat ratio, osteopenia, fatigue, muscle weakness, reduced extracellular fluid volume, and decreased cardiac function *(3)*. Because these clinical deficits in GHDA are largely normalized with GH therapy, it has been proposed that restoration of the GH/IGF-1 axis to a young adult profile could prevent or reverse much of the progressive physical and functional decline that occurs with normal aging.

The decrease in GH levels with aging is largely due to reduction in the amplitude and duration of GH pulses such that the fraction of GH secreted in pulses is diminished while pulse frequency remains unchanged *(4)*. Although recombinant GH therapy represents the current standard of care for GH-deficient patients, it is not ideal because of side effects, parenteral delivery, and high cost typical of protein therapeutics. Side effects such as carpal tunnel syndrome, hypertension, fluid retention, and arthralgias have been linked to the unnatural pattern of hormone replacement produced by injection of the recombinant protein *(5,6)*. Administration of GH secretagogues (GHSs) has been shown to augment GH pulse amplitudes and integrated hormone production in elderly subjects *(7–10)*. Chronic studies with the peptidomimetic GHSs L-692,429 and MK-0677 have been particularly important in demonstrating that secretagogues selectively amplify the normal pulsatile profile of GH secretion without disrupting or overriding physiologic feedback regulation of hormone levels. These findings suggest that a viable alternative approach to restoration of GH levels in the elderly is by treatment with a secretagogue.

One pharmacologically important class of GHSs is based on the activity of growth–hormone releasing peptide-6 (GHRP-6) *(11)* and comprises a structurally diverse group of molecules that share a common mechanism for stimulating GH release. Compounds in this class act via a novel

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G-protein-coupled receptor designated GHS-R to elevate intracellular calcium, increase phosphoinositol turnover, and activate protein kinase C *(12)*. Early efforts by scientists from Merck and Genentech proved successful in significantly reducing the size of the active pharmacophore *(13,14)*. Achievement of oral activity in the small peptidomimetic MK-0677 represented another critical breakthrough for pharmaceutical research on synthetic GHSs *(15)*.

CP-424,391 391 belongs to a novel series of orally active, pyrazolinone-piperidine dipeptide GHSs (**Fig. 1**). It was identified following the comparison of peptidomimetic GHS structures discovered by Merck with a series of active tetrahydroquinolines possessing potent activity but low oral bioavailability *(16)*. The intrinsic GH- releasing activity of these novel structures was first determined in cultures of rat pituitary cells, and then in vivo activity was evaluated in rat and dog models. The GHS 391 is the most extensively characterized compound from this series and has been selected for clinical evaluation based on its potent activity and excellent oral bioavailability in preclinical species.

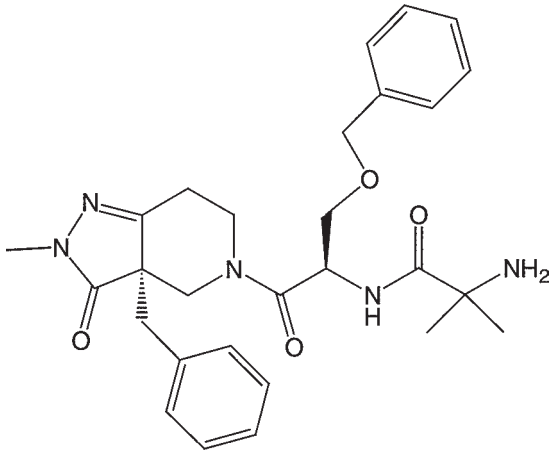

Fig. 1. Structure of CP-424,391 (**391**).

Results

In Vitro Activity

The addition of 391 rapidly stimulated GH release from rat pituitary cell cultures in a dose-dependent manner. The maximal stimulation of GH levels in culture medium was three- to fourfold in a 15-min assay with an EC_{50} = 3 n*M* (**Fig. 2**). The magnitude of GH response produced by **391** was comparable to that produced by 100 n*M* GHRP-6, and the addition of a maximally efficacious concentration of GHRP-6 together with 1 μM 391 produced no further augmentation of GH release (**Fig. 3**). By contrast, the addition of 10 n*M* growth hormone–releasing hormone (GHRH) alone showed less than two-fold stimulation of GH release in the standard 15-min assay, but the combination of 10 n*M* GHRH with 1 μM 391 elicited a markedly greater degree of GH secretion than either single agent. Pretreatment with 100 n*M* GHRP-6 for 1 h completely abolished any subsequent response to 391; however, pretreatment with 10 n*M* GHRH roughly doubled the amount of GH released by 391 compared with the amount released by cultures receiving no pretreatment (**Fig. 4**). These findings suggest that 391 shares its mechanism of GH release with GHRP-6.

Because GHRP-6 is known to activate intracellular calcium signaling *(17),* we examined the effect of **391** on intracellular calcium concentration in cells isolated from rat anterior pituitary glands. The percentage of somatotrophs in these cultures was 30–50%, based on immunohistochemical analysis using anti-rat GH antisera. Using a fluorescence videomicroscopy imaging system, we monitored the responses of fura-2-loaded pituitary cells to superfusion with 0.3, 3, or 30 n*M* 39**1** (**Fig. 5**). All concentrations elicited a rapid and transient increase in intracellular calcium concentration in a discrete subset of cells. The percentage of responding cells was approximately one-third of total cells at all three concentrations of 391; however, the magnitude of calcium flux clearly increased between 0.3 and 3 n*M* and then appeared to plateau. A brief superfusion with either 391 or GHRP-6 rendered all cells refractory to subsequent stimulation with 391 (**Fig. 6**). Tachyphylaxis of the calcium response appeared restricted to the GHS signaling pathway since the same cells showed elevation of intracellular calcium upon depolarization with 60 m*M* KCl, and parallel cultures repeatedly exhibited calcium fluxes when subjected to multiple cycles of depolarization-repolarization using KCl.

The discovery of a G-protein-coupled cell-surface receptor possessing high affinity for GHRP-6 and MK-0677 provided a physiologic target for this class of GH-releasing agents *(12,18)*. This receptor is distinct from the receptor for GHRH and has been designated GHS-R. Its ligands include GH-releasing peptides and nonpeptidyl GH secretagogues. To determine whether 391 is a ligand for GHS-R, we conducted competitive binding assays using a membrane fraction prepared from HEK293 cells that had been engineered to express recombinant GHS-R. Under conditions where unlabeled MK-0677 could inhibit binding of 400 p*M* [^{35}S]MK-0677 with an IC_{50} = 3 ± 1 nM, 391 competed for radioligand binding with an IC_{50} = 18 ± 7 n*M*. Transfection with a plasmid-encoding recombinant human GHS-R allowed nonpituitary cell lines, such as the hamster pancreatic cell line HIT-T15 (HIT), to respond to 391 with intracellular calcium fluxes. Untransfected HIT cells responded to bombesin but not to 391 (**Fig. 7A**). In four independent experiments, transient transfection with human GHS-R cDNA resulted in 391-induced intracellular calcium signaling in 4/14, 7/14, 5/8, and 6/20 cells (**Fig. 7B**). In a similar manner, stable transfection of GHS-R DNA conferred 391 responsiveness onto clonal derivatives of the HEK293 human embryonic kidney line (data not shown).

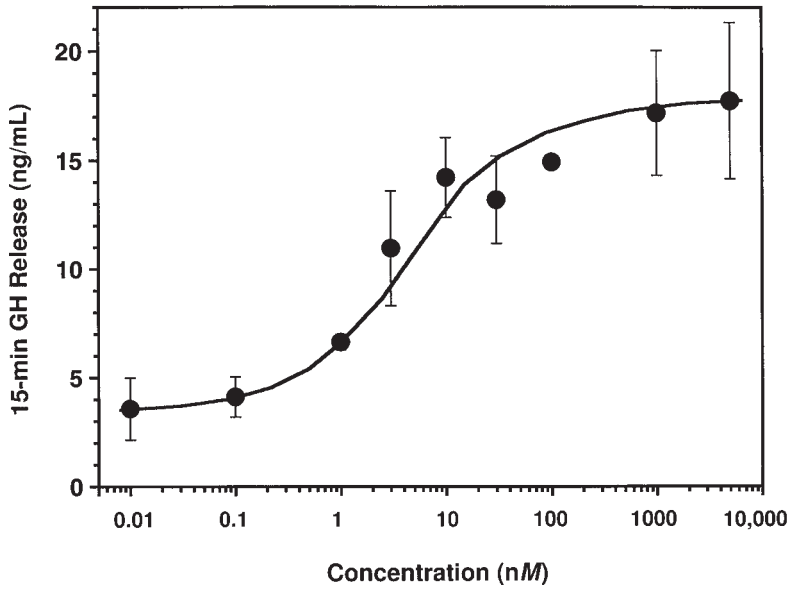

Fig. 2. GH release from rat pituitary cells treated with increasing concentrations of 391.

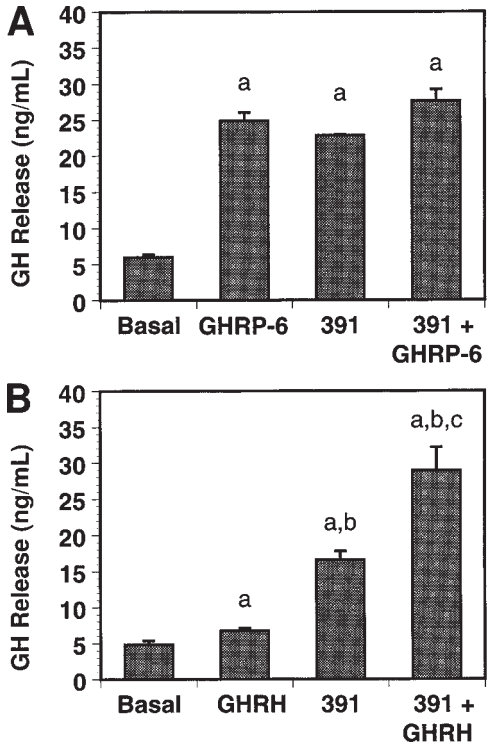

Fig. 3. GH release from pituitary cells treated simultaneously with 1 μ*M* 391 plus (**A**) 100 n*M* GHRP-6, replicates of four wells, or (**B**) 10 n*M* GHRH, replicates of eight wells. Data in panels (**A**) and (**B**) are from separate experiments. In each experiment, GH release by the individual agents was also determined. $^{a}p < 0.05$ vs basal; $^{b}p < 0.001$ versus GHRH; $^{c}p < 0.05$ vs 391.

The basic pulsatile rhythm of GH secretion is thought to be largely governed by the interplay of two hypothalamic peptides, GHRH and somatostatin *(19).* Therefore, ability of 391 to modulate GH release and intracellular signaling by GHRH and somatostatin was examined in primary pituitary cell cultures. For these studies, cells were incubated

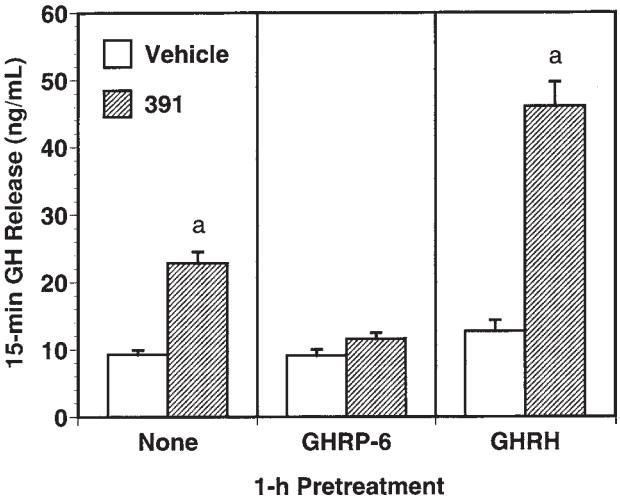

Fig. 4. Effect of pretreatment with GHRP-6 or GHRH on the subsequent GH release elicited by 391. Cultures were equilibrated in the presence of 100 n*M* GHRP-6 or 10 n*M* GHRH for 1 h. Following removal of the equilibration medium, either vehicle or 1 μ*M* 391 was added only during the 15-min release period. $^{a}p < 0.005$ vs vehicle for the corresponding pretreatment.

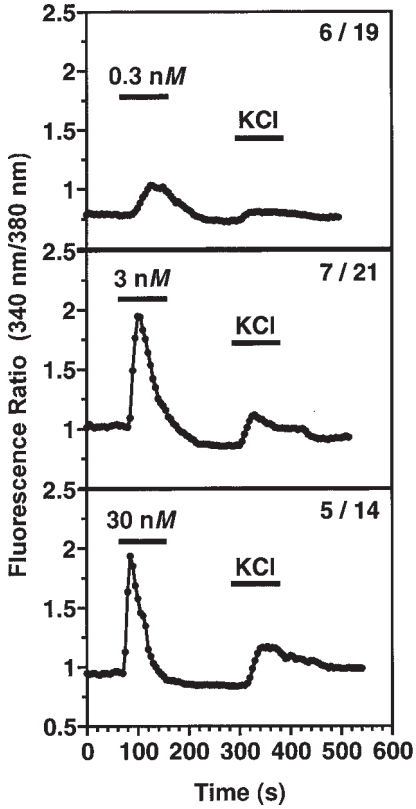

Fig. 5. Fluorescence ratio imaging of intracellular calcium in fura-2-loaded rat pituitary cells. Each graph is a single experiment showing the average fluorescence profile of only the 391-responsive cells. Superfusion with 391 at the indicated concentration (first bar) was followed by a 2-min washout with KRH buffer, then application of 60 m*M* KCl (second bar). The fraction in the upper right of each panel indicates the number of cells that responded to 391 (numerator) over the total number of cells analyzed (denominator).

with control medium or medium containing 20 n*M* somatostatin-14 for 1 h prior to a 15-min test period during which 391, GHRH, or both GH releasers were added. The results shown in **Fig. 8** have been reproduced in multiple experiments. Somatostatin tended to suppress both basal and stimulated GH release. 391 alone did not elevate cyclic adenosine monophosphate (cAMP) and its stimulation of GH release was partially attenuated by somatostatin. GHRH elevated both cAMP and GH release, and its responses were completely blocked by somatostatin. Both cAMP accumulation and GH release in response to GHRH were potentiated by 391. Even in the presence of inhibitory concentrations of somatostatin, the concomitant addition of 391 and GHRH produced a significant stimulatory effect on both cAMP and GH release. Collectively, these results suggest that 391 enhances the activity of GHRH and functionally opposes the inhibitory effects of somatostatin on GHRH in pituitary somatotrophs.

Because elevation of plasma adrenocorticotropic hormone (ACTH) and cortisol levels has been associated with the GH response to acute systemic administration of GHSs, we examined the ability of 391 or GHRP-6 to stimulate ACTH release in mixed pituitary cell cultures. Both GH and ACTH were measured in the same culture supernatants after 15 min of stimulation with GHRP-6, 391, $(Arg)^8$-vasopressin (AVP), corticotropin-releasing factor (CRF), or combinations of these agents (**Table 1**). In the standard 15-min release assay, CRF and AVP synergistically stimulated ACTH release with little effect on GH. By contrast, 391 at concentrations up to 1 μ*M* failed to elevate ACTH above basal levels. GHRP-6 slightly augmented ACTH release at 100 n*M* in one of two experiments. The addition of 391 together with a submaximal concentration of either AVP or CRF had no effect on the release of ACTH; conversely, concurrent treatment with AVP and CRF did not alter the GH-releasing activity of 391.

In Vivo Activity

We assessed the in vivo GH-releasing activity of 391 in a pentobarbital-anesthetized rat model. Pentobarbital has been shown to suppress hypophyseal portal somatostatin levels, which can interfere with the ability of secretagogues to induce GH release *(20)*. In this model, iv injection of 391 (3.9 mg/kg) elicited a maximal response, rapidly stimulating serum GH levels from 92 ± 13 ng/mL to a peak of 992 ± 165 ng/mL (**Fig. 9A**). The ratio of the serum GH concentration at 10 min to the GH level prior to injection was dose related and gave an ED_{50} of approximately 0.04 mg/kg (**Fig. 9B**).

The chronic efficacy of 391 was evaluated by its effect on body weight in subadult and middle-aged female rats. To assess its effect in growing animals, 391 at 3.9 mg/kg or vehicle (water) was administered daily for 28 days to

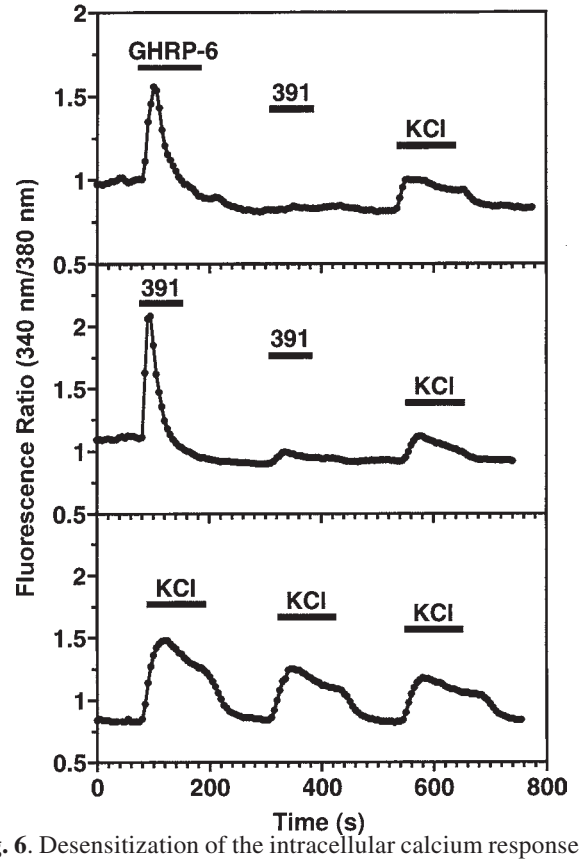

Fig. 6. Desensitization of the intracellular calcium response following stimulation by 391 or GHRP-6. Rat pituitary cells were loaded with fura-2 and subjected to fluorescence ratio imaging to measure changes in intracellular calcium during superfusion. Each panel represents a separate experiment in which three sequential stimuli were applied in the superfusion buffer, each followed by a 2-min washout with buffer alone. The calcium profile shown is the average of all cells that responded to the first stimulus.

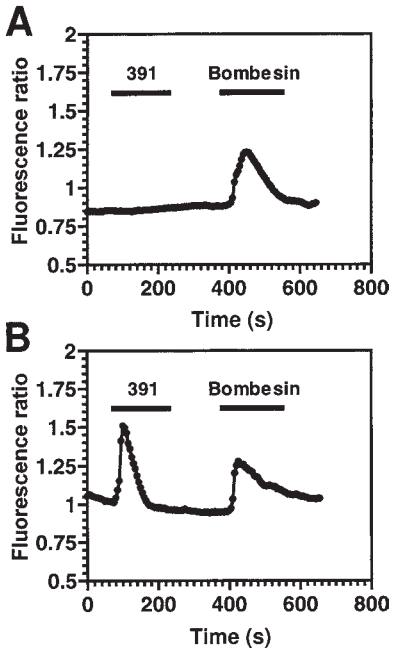

Fig. 7. Effect of bombesin and 391 on calcium signaling in HIT-T15 cells. Cells were loaded with fura-2 and subjected to fluorescence ratio imaging to measure changes in intracellular calcium during superfusion. Each panel is the average fluorescence profile of all cells in a single experiment. (**A**) Untransfected HIT cells; (**B**) HIT cells 48 h after transfection with GHS-R.

Table 1
Effect of 391, CRF, and AVP on GH and ACTH Release by Rat Pituitary Cultures

Treatment	GH (ng/mL)	ACTH (pg/mL)
Basal	40 ± 7d	32 ± 13
1 μ*M* 391	121 ± 20 [a–c]	32 ± 17
1 n*M* CRF	48 ± 4 [d]	57 ± 18
1 n*M* CRF + 1 μ*M* 391	109 ± 5 [a–c]	59 ± 14[a]
10 n*M* AVP	48 ± 8[d]	39 ± 19
10 n*M* AVP + 1 μ*M* 391	114 ± 20 [a–c]	30 ± 15
1 n*M* CRF + 10 μ*M* AVP	49 ± 9[d]	84 ± 22[a]

[a] $p < 0.05$ vs basal..
[b] $p < 0.05$ vs 1 n*M* CRF.
[c] $p < 0.05$ vs 10 n*M* AVP.
[d] $p < 0.05$ vs 1 μ*M* 391.

7-wk-old female rats by oral gavage. Vehicle-treated rats at this age grew rapidly; however, 391 further augmented weight gain. The difference between average body weight in the two groups first reached significance ($p < 0.05$) after 15 d of treatment (**Fig. 10**). The average weight increase for the 391 group over this 4-wk interval was 83 g (43.6%) compared with 64 g (33.5%) for the placebo-treated group. Body composition analysis at the beginning and end of the

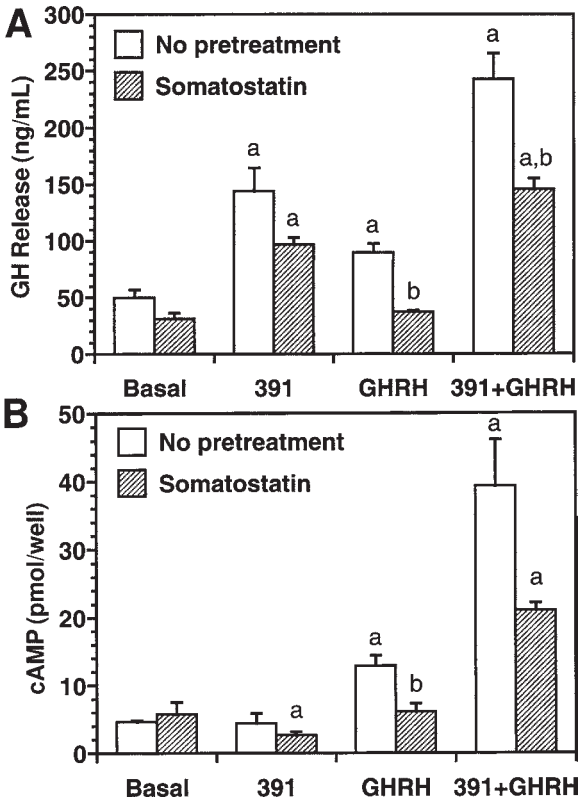

Fig. 8. Effect of somatostatin on the stimulation of GH release and cAMP production by 391 and GHRH in rat pituitary cells. Somatostatin (20 n*M*) was added to one set of cultures during both the 1-h equilibration period and 15-min release assay. During only the release assay, 100 n*M* 391 and 10 n*M* GHRH or both agents were added. (**A**) GH release, (**B**) cAMP levels. $^{a}p < 0.05$ versus basal conditions (without somatostatin), $^{b}p < 0.05$ for effect of somatostatin on each treatment.

treatment period showed no differences between vehicle and treated rats in whole-body percentages of fat, lean mass, or bone content by unpaired Student's *t*-test, indicating that 391 enhanced growth in a proportional manner (**Table 2**). A comparable growth effect was observed in middle-aged (10-mo-old) female rats treated with the same dose by the same route of administration. After 3 mo of treatment, the placebo-treated older rats had lost an average of 11 g (–2.2% of their pretreatment body weight) whereas age-matched 391-treated rats had gained 131 g (+27.8% of their pretreatment body weight).

Plasma GH and cortisol levels were measured in four fasted beagle dogs following a single oral dose of 391 administered in the morning. All four dogs responded to 1 mg/kg with a peak in plasma GH levels at 30 min, whereas only three of four responded to 0.1 mg/kg, and two of four responded to 0.05 mg/kg (**Fig. 11**). The 0.01 mg/kg dose failed to elicit significant GH pulses. In all dose groups, GH levels returned to baseline by 8 h after dosing. The ED_{50} for integrated GH secretion for 4 h after dosing was estimated to be 0.3 mg/kg. Plasma cortisol was transiently elevated to 8.6 ± 1.4 mg/dL (mean ± SEM) within 1 to 2 h of a single oral 1 mg/kg dose (**Fig. 12**) and returned to baseline by 4 h. At doses below 0.5 mg/kg, cortisol levels did not exceed the peaks observed in placebo-treated dogs (5.8 and 4.3 µg/dL). During the first 2 h after dosing. There were no obvious gender differences in either GH or cortisol responses.

Plasma GH and IGF-I levels were measured in a 5-d study of once daily oral dosing at 1 mg/kg to beagle dogs. Chronic dosing produced attenuation of the GH response in conjunction with elevation of plasma IGF-1 levels. The mean amplitude of GH peaks (±SEM) decreased from 66 ± 24 ng/mL on day 1 to 28 ± 16 ng/mL on d 5 (**Fig. 13A,B**). On study d 1, 2, and 5, plasma IGF-I levels measured 8 h after dosing were increased by 57, 109, and 96% over baseline whereas the 24-h IGF-I levels showed a progressive increase of 12, 39, and 57%, respectively (**Fig. 13C**). There was no evidence of IGF-I elevation in the placebo-treated group. In studies of longer duration, the maximal change in IGF-I was dose dependent and occurred after 1 wk of daily dosing but remained significantly elevated for the entire duration of treatment. Chronic treatment for 12 mo with doses as low as 0.3 mg/kg showed a trend toward increased body weight.

Discussion

Initial studies demonstrated the GH-releasing activity of **391** in isolated pituitary cells and intact animal models, but did not define its pharmacologic target or mechanism of

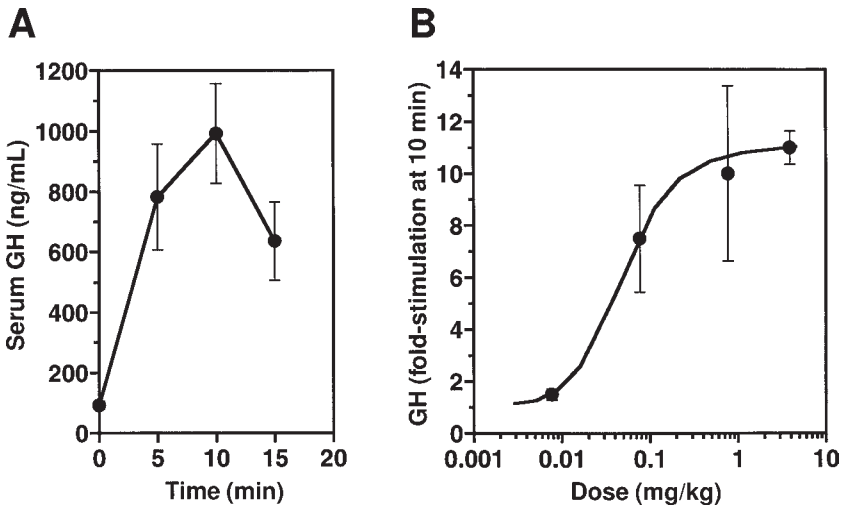

Fig. 9. Serum GH levels after iv administration of 391 to pentobarbital-anesthetized rats. Four-week-old female rats were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, intraperitoneally), and then a baseline blood sample was collected by tail nick (0 min). Blood samples were collected by tail nick at the indicated times after injection of 391 into the tail vein. Each data point is the mean ± SEM for three rats per dose. (**A**) Temporal profile of serum GH levels after injection of 3.9 mg/kg of 391. (**B**) relationship of GH pulse amplitude to dose at 10 min.

Table 2
Effect of 4 wk of Oral 391 Treatment
on Body Composition in Subadult Female Rats.[a]

	Day 1		Day 29	
Total body mass (%)	Vehicle	391	Vehicle	391
Fat mass (%)	13.3 ± 1.0	12.9 ± 0.8	15.9 ± 1.3	17.1 ± 1.1
Lean mass (%)	84.4 ± 1.0	84.9 ± 0.9	81.6 ± 1.2	80.4 ± 1.1
Bone mass (%)	2.30 ± 0.03	2.22 ± 0.03	2.55 ± 0.05	2.46 ± 0.04

[a]Female Sprague-Dawley rats were administered 391 (3.9 mg/kg) or vehicle once daily by oral gavage for 28 d beginning at the age of 7 wk. Body composition was assessed by dual X-ray absorptiometry (Hologic QDR/1000W equipped with whole-body composition software; Waltham, MA) at the beginning and end of the treatment period.

action. Subsequent in vitro studies clearly showed 391 to be a GHRP-6 mimetic whose activity resembled that of L-692,429 and MK-0677 but not GHRH. In mixed pituitary cell cultures, 391 activated calcium-mediated but not cAMP-mediated signal transduction and increased GH but not ACTH secretion. The percentage of responsive pituitary cells corresponded roughly to the percentage of somatotrophs identified by immunolocalization of GH. Following the molecular cloning of the GHS-R, a new G-protein-coupled receptor that serves as a specific target for peptide and nonpeptidyl GHSs, it was possible to show that 391 bound to and activated this receptor. The high binding affinity and potent agonist activity of 391 at recombinant human GHS-R contrasts with its failure to interact with a panel of more than 40 known neurotransmitter receptors and uptake sites, nuclear hormone receptors, ion channels, peptide hormone receptors, and other regulatory sites. Although evidence exists for multiple classes of binding sites for some GHSs *(21,22)*, the ability of 391 to interact with these other receptor subtypes is not presently known. Nevertheless, our data suggest that the cells activated by 391 in the pituitary are somatotrophs and demonstrate convincingly that one molecular target of 391 is the GHS-R.

Downstream of 391 binding to its receptor in pituitary somatotrophs, there appears to be crosstalk with other signal transduction pathways that may not be directly linked to GH exocytosis. Although 391 alone is unable to increase intracellular cAMP levels, 391 amplified the cellular accumulation of cAMP stimulated by GHRH, even in the face of inhibitory signaling by somatostatin. Similar findings have been reported for GHRP-6 in rat pituitary cells *(23)* and on human pituitary somatotropinomas with adenylyl cyclase-activating *gsp* oncogenes *(24)*. One possible explanation is that GHSs modulate adenylyl cyclase activity downstream of phosphoinositol turnover and protein

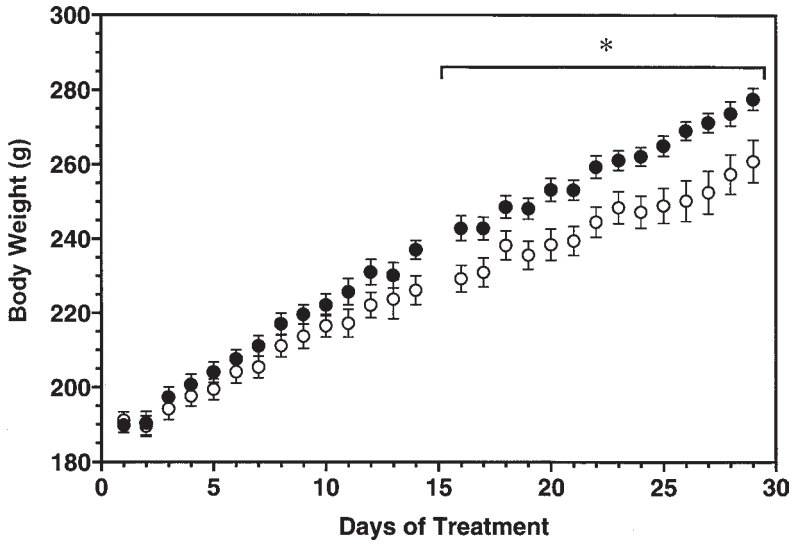

Fig. 10. Body weight changes during a 4-wk oral study of 391 in subadult female rats. Seven-week-old female Sprague-Dawley rats were dosed daily by oral gavage with 3.9 mg/kg of 391 (●) or vehicle (water, ○) and weighed. Data are mean ± SEM for 10 rats per treatment group. *$p < 0.01$ vs vehicle by unpaired student's *t*-test.

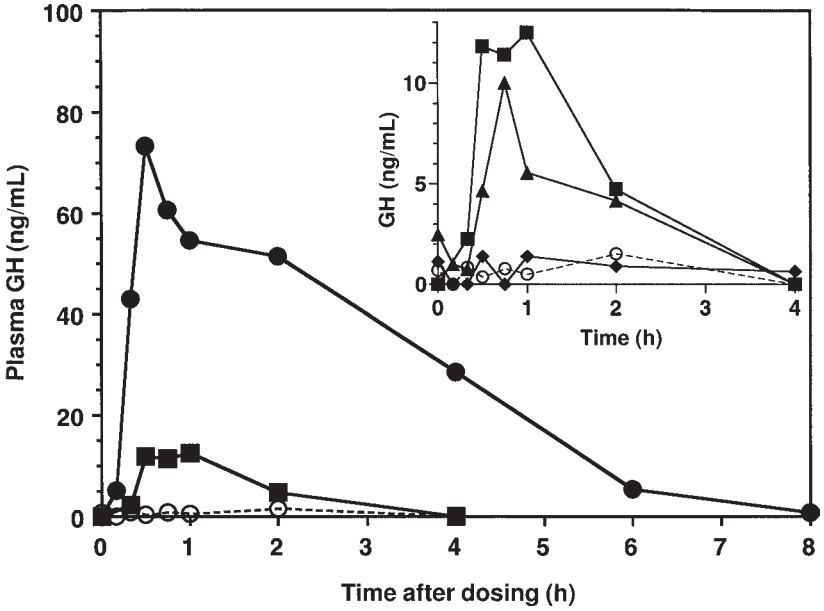

Fig. 11. Plasma GH levels after acute oral dosing of 391 in dogs. Data are averages for four dogs (two males and two females) per group except for the vehicle group, which consisted of seven dogs (five males and two females). Plasma GH values showed log-normal distributions (error bars are omitted for the sake of clarity). Data for the lower doses are plotted on the inset graph with an expanded vertical scale. ○, vehicle; ◆, 0.01 mg/kg; solid triangles ▲, 0.05 mg/kg; ■, 0.1 mg/kg; ●, 1 mg/kg.

kinase C activation. An alternative possibility is that signaling via the GHS-R inhibits phosphodiesterase activity, which would slow the destruction of a cAMP signal produced by GHRH receptor activation. In addition to its inhibitory coupling to adenylyl cyclase, somatostatin hyperpolarizes cells by increasing potassium ion conductance and consequently decreasing intracellular free calcium *(25)*. It has been proposed that GHSs functionally antagonize somatostatin by blocking potassium currents, leading to depolarization and calcium influx via voltage-gated L-type calcium channels *(19)*. However, further studies are required to clarify the precise mechanisms of crosstalk between these particular hormone receptors and their signal transduction pathways.

Elevation of plasma IGF-I and increased body weight gain following chronic dosing with 391 constitute evidence for persistent activation of the GH/IGF-I endocrine axis. Despite attenuation of GH pulses with chronic dosing, augmentation of integrated 24-h GH secretion by amplification of the normal pulsatile patterns was observed after

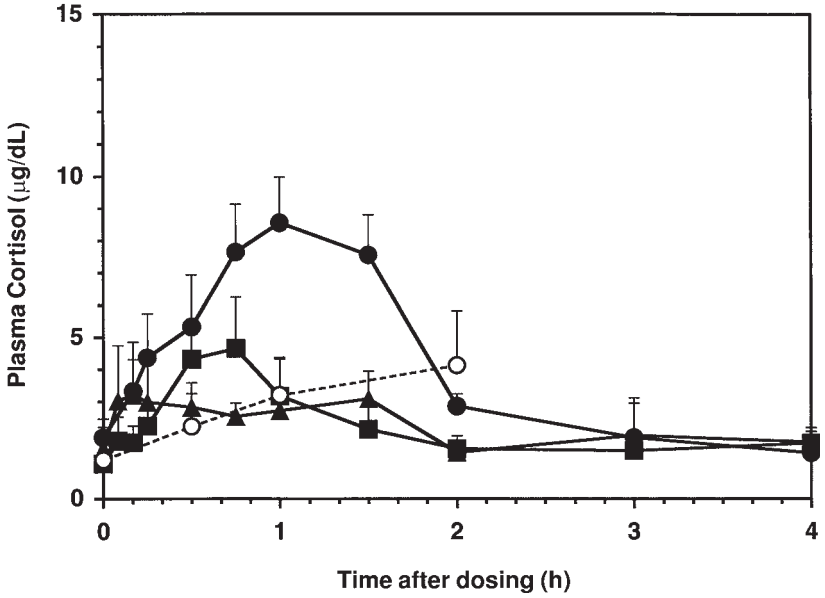

Fig. 12. Plasma cortisol levels after acute oral dosing of 391 in dogs. Data are mean ± SEM for four dogs (two males and two females) per group except for the vehicle group, which consisted of two dogs (one male and one female). ○, vehicle; ▲, 0.1 mg/kg; ■, 0.5 mg/kg; ●, 1 mg/kg. (Error bars are shown in the plus direction only for the sake of clarity.)

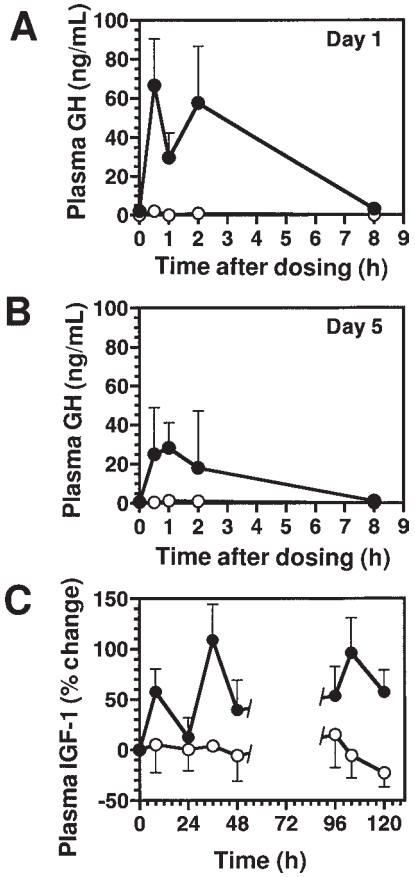

Fig. 13. Plasma GH and IGF-1 levels during a 5-d oral study of 391 in dogs. Data are mean ± SEM for four dogs (two males and two females) per group. ○, vehicle; ●, 1 mg/kg 391. (**A**) GH levels on d 1; (**B**) GH levels on d 5; (**C**) IGF-1 levels.

2 wk of daily treatment with MK-0677*(26)*. The increase in plasma IGF-I also indicates that elevanted circulating levels of GH have activated physiologic feedback mechanisms. Although the enhancement of body weight gain in 391-treated animals is consistent with a GH-mediated mechanism of action, some GHRPs are known to stimulate food intake *(27,28)*. In our studies, food intake was neither controlled nor measured. If 391 stimulated feeding behavior in addition to GH secretion, both mechanisms could have contributed to the observed growth effect.

Recent clinical studies have made significant strides toward the demonstration on that GHSs can prevent or treat somatopause and the attendant musculoskeletal frailty. MK-0677 prevented diet-induced nitrogen loss in healthy volunteers *(29)*, increased biochemical markers of bone turnover in young adult and elderly subjects *(30,31),* and increased fat-free mass *(32)*. MK-0677 may also improve sleep quality *(33)*. In dogs, MK-0677 accelerated recovery of muscle mass and strength during rehabilitation following 10 wk of hindlimb immobilization *(34)*. Hexarelin, a peptide GHS, has been shown to acutely increase left ventricular ejection fraction in normal and hypopituitary subjects *(35)* and has demonstrated cardioprotective effects in an experimental model of postischemic ventricular dysfunction *(36–38)*.

The therapeutic potential of 391 has been suggested by studies in animal models of two chronic degenerative diseases associated with aging, osteoporosis and dilated cardiomyopathy. Our hypothesis that the bone anabolic action of a GHS could be enhanced by an antiresorptive agent was supported by a study in which 391 was administered alone or in combination with the selective estrogen receptor modulator lasofoxifene to osteopenic ovariectomized rats. In this study, 4 wk of combination therapy produced a larger increase in femoral bone mass than either 391 or lasofoxifene alone *(39)*. 391 was also tested in a swine model of tachycardia-induced congestive heart failure following the demonstration that GH could slow disease progression in this model *(40)*. Like GH, 391 was able to produce left ventricular hypertrophy and partially mitigate the deterioration of cardiac pump function and cardiomyocyte contractile function caused by 3 wk of pacing overdrive *(41)*. Further clinical studies will be required to fully evaluate the utility of GHSs to prevent or treat of the physical frailty of aging.

Materials and Methods

Animals

Wistar and Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were vivarium housed with a 12-h light/12-h dark cycle and provided free access to standard rodent chow (Agway Prolab RMH 3000 or Purina Rodent Diet 5001) and water. Beagle, male and female, 2 to 5-yr old, were purchased from Marshall Farms (North Rose, NY). Dogs were fasted overnight, dosed in the early morning by oral gavage, and then fed approx 3 h after dosing. Access to water was provided at all times. Canine blood samples were withdrawn by direct venipuncture of the jugular vein into heparinized vacutainers. Serum and plasma samples were stored frozen pending hormone assays. All animal studies were conducted under approved protocols in accordance with National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Bioactive Peptides

GHRP-6, rat GHRH, ovine somatostatin-14, bombesin, AVP, and rat CRF were purchased from Bachem (Torrance, CA). All peptides were initially dissolved in 10% ethanol, 10 m*M* acetic acid, and 0.1% bovine serum albumin (BSA) and then diluted into release medium.

Rat pituitary Cell Cultures and Hormone Release Assays

Primary pituitary cell cultures were established by enzymatic dissociation of anterior pituitary glands from 6 wk old male Wistar rats by an adaptation of published protocols *(23)*. Typical cell yields were approximately 10^6 viable cells/gland based on Trypan Blue exclusion. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L of glucose and supplemented with 1 m*M* sodium pyruvate, 1% Minimal Essential Medium nonessential amino acids, 10% heat-inactivated horse serum, and 2.5% fetal bovine serum and antibiotics, plated at 1×10^5 cells/well in 24-well tissue culture plates (Costar, Corning, NY) and incubated in a humidified 5% CO_2/95% air incubator at 37°C. Hormone release and calcium-imaging experiments were performed 3 to 4 d after plating. For hormone release assays, cell cultures were rinsed twice and then equilibrated at 37°C in release medium (DMEM with 25 m*M* HEPES buffer, pH 7.4, and 5 mg/mL of BSA) for 30 min unless otherwise indicated. This medium was aspirated and replaced with prewarmed release medium containing test agents. After 15 min of incubation at 37ºC, the medium was removed and assayed for GH or ACTH as described in the next section. Results are expressed as mean ± SEM of quadruplicate wells. Groups were compared by unpaired Student's two-tailed *t*-test unless otherwise indicated.

Biochemical Assays

Rat and canine GH were measured by species-specific double antibody radioimmunoassays (RIAs) using reagents and protocols obtained from the NIDDK National Hormone and Pituitary Program (Dr. A. F. Parlow, Harbor–UCLA Medical Center, Torrance, CA). Aliquots of rat and canine GH were labeled with iodine-125 using chloramine T. For both assays, immune complexes were precipitated using goat anti-monkey IgG (Cappel no. 55418; Organon Teknika, Durham, NC) in the presence of 7% polyethylene glycol (Mol wt 15,000–20,000). Plasma samples were extracted with acid ethanol (0.25 N HCl in 90% ethanol) and centri-

fuged, and then the supernatant was neutralized with Tris base prior to determination of IGF-I concentration using the IGF-I by extraction kit (Nichols Institute, San Juan Capistrano, CA). ACTH was measured using a double-antibody RIA kit (No.07-106101; ICN, Orangeburg, NY). Canine plasma cortisol was measured using the Coat-A-Count Cortisol kit (Diagnostic Products, Los Angeles, CA), which had been previously validated for this species. Cell lysates for cAMP were prepared by lysing cells in 0.1 *N* HCl, neutralizing with NaOH, and centrifuging to remove insoluble matter then assayed using a cAMP radioimmunoassay (NEK# 033; New England Nuclear, Boston, MA).

Intracellular Calcium Imaging

For fluorescence ratio imaging of intracellular calcium, cells were plated onto poly-***D***-lysine-coated glass cover slips at 1 to 1.25×10^5 cells/cm^2. Cells were loaded with 5 µ*M* fura-2 AM (Molecular Probes, Eugene, OR) in a Krebs-Ringers HEPES buffer (KRH) (140 m*M* NaCl, 5 m*M* KCl, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 1.2 m*M* KH_2PO_4, 25 m*M* HEPES, 6 m*M* glucose, pH 7.4) for 30 min at room temperature, then mounted in a superfusion chamber on the stage of a Zeiss Axiovert 135 epifluorescence microscope. Cells were continuously superfused at a flow rate of 1 mL/min at room temperature with KRH buffer. All test substances were diluted into KRH for superfusion. One selected microscope field on each cover slip was alternately excited at wavelengths of 340 and 380 nm for the duration of the experiment. Regions corresponding to single cells in a field were digitally circumscribed for data collection and analysis. Emission signals at 510 nm were collected simultaneously at 5-s intervals from all cells in the field using a low-light charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan). Images were digitized, and the fluorescence ratio for the two excitation wavelengths (340 and 380 nm) was calculated and stored on-line for each selected region in the field of view. This fluorescence ratio is proportional to the free calcium concentration. Data was analyzed using Videoprobe software.

Cell Lines

Human embryonic kidney-derived HEK293 and hamster pancreatic islet HIT-T15 lines were obtained from the American Type Culture Collection. Transfections were performed using Lipofectamine (Life Technologies, Gaithersburg, MD) as per the manufacturer's instructions. Human GHS-R cDNA was amplified by reverse transcriptase-polymerase chain reaction and cloned into the mammalian expression vector pZeoSV2(+) (Invitrogen, Carlsbad, CA.)

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