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Synthesis and biological evaluation of antagonists of growth hormone-releasing hormone with high and protracted *in vivo* activities

(inhibitors of GH release/structure-activity relationships/cancer therapy)

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ABSTRACT **Some antagonists of human growth hormone-releasing hormone (hGH-RH) synthesized previously were shown to inhibit *in vivo* proliferation of various human cancers in nude mice. However, the activity of these analogs requires an increase to assure clinical efficacy. In an attempt to prepare hGH-RH antagonists with a high and protracted activity, we synthesized and biologically tested 22 antagonistic analogs of hGH-RH(1–29)NH_2. The ability of the antagonists to inhibit hGH-RH-induced GH release was evaluated *in vitro* in a superfused rat pituitary system, as well as *in vivo* after i.v. injection into rats. The binding affinity of the peptides to GH-RH receptors also was determined. All antagonistic analogs had the common core sequence [PhAc-Tyr^1,D-Arg^2, Phe(4-Cl)^6 (*para*-chlorophenylalanine), Abu^{15} (α-aminobutyric acid),Nle^{27}]hGH-RH(1–29)NH_2 and contained Arg, D-Arg, homoarginine (Har), norleucine (Nle), and other substitutions. The following analogs were determined to have a high and/or protracted antagonistic activity: [PhAc-Tyr^1,D-Arg^2,Phe(4-Cl)^6,Arg^9,Abu^{15},Nle^{27},D-Arg^{29}]hGH-RH(1–29)NH_2 (JV-1–10), [PhAc-Tyr^1,D-Arg^2,Phe(4-Cl)^6,Abu^{15},Nle^{27},D-Arg^{28},Har^{29}]hGH-RH(1–29)NH_2 (MZ-6–55), [PhAc-Tyr^1,D-Arg^2,Phe(4-Cl)^6,Arg^9,Abu^{15},Nle^{27},D-Arg^{28},Har^{29}]hGH-RH(1–29)NH_2 (JV-1–36), and [PhAc-Tyr^1,D-Arg^2,Phe(4-Cl)^6, Har^9,Tyr(Me)^{10},Abu^{15},Nle^{27},D-Arg^{28},Har^{29}]hGH-RH(1–29)NH_2 (JV-1–38). Among the peptides tested, analog JV-1–36 showed the highest GH-RH antagonistic activity *in vitro* and also induced a strong and prolonged inhibition of GH release *in vivo* for at least 30 min. The antagonist JV-1–38 was slightly less potent than JV-1–36 both *in vitro* and *in vivo* but proved to be very long-acting *in vivo*, suppressing the GH-RH-induced GH release even after 60 min. High and protracted *in vivo* activities of these antagonists indicate an improvement over earlier GH-RH analogs. Some of these hGH-RH antagonists could find clinical applications in the treatment of cancers dependent on insulin-like growth factors I and II.**

Since the isolation and structural elucidation of human growth hormone-releasing hormone (hGH-RH), various analogs of GH-RH have been synthesized (1–11). Most of them were agonists intended for clinical and veterinary applications (5–8), but there is a greater medical need for antagonistic analogs of GH-RH (12–14). GH-RH antagonists may find use in conditions such as acromegaly, diabetic retinopathy, or diabetic nephropathy (glomerulosclerosis). However, the main applications of GH-RH antagonists would be in the field of cancer (12, 14, 15), in view of their ability to inhibit the production of insulin-like growth factors I and II (IGF-I and -II). Both IGF-I and IGF-II have been implicated in malignant transformation of cells, tumor progression, and metastases of various cancers (12, 14–24). By suppressing GH secretion, GH-RH antagonists decrease the synthesis of IGF-I in the liver and other tissues and reduce serum IGF-I levels (20–24). That autocrine or paracrine production of IGF-I by various tumors (17) is also under the control of GH is suggested by inhibition of tumor IGF-I levels by GH-RH antagonists (20–23). GH-RH antagonists can likewise suppress the production and mRNA expression of IGF-II in diverse tumors (21–24). Thus, inhibition of growth of various experimental tumors by GH-RH antagonists can be linked to a reduction of IGF-I and IGF-II levels or their secretion (14, 15, 20–24).

It was established that the shortest sequence of hGH-RH that retains essentially full GH releasing activity resides in the GH-RH(1–29) peptide (1). Accordingly, this sequence has been used for development of agonistic and antagonistic GH-RH analogs (2–11). Replacement of the naturally occurring Ala^2 residue by D-Arg^2 accounts for the antagonistic property of [Ac-Tyr^1,D-Arg^2]hGH-RH(1–29)NH_2, the first described GH-RH antagonist (2). This peptide, termed herein as the "standard antagonist," inhibits the GH-RH-stimulated adenylate cyclase activity in rat pituitary cells (2), reduces GH release in cell cultures (4), and blocks endogenous and GH-RH-induced GH secretion in rats (25, 26). Subsequent studies confirmed the essential role of D-Arg^2 substitution for generating GH-RH antagonistic activity (3–5). Given the preponderant α-helical amphiphilic nature of GH-RH (6, 27), many attempts were made to stabilize this helical structure and enhance its amphiphilicity in GH-RH analogs (5–11). Both GH-RH agonistic (5–8) and antagonistic (5, 9–11) peptides with increased biological activities were produced in this way.

Previously, researchers in our group reported the synthesis of various antagonists, including [PhAc-Tyr^1,D-Arg^2, Phe(4-Cl)^6 (*para*-chlorophenylalanine),Abu^{15} (α-aminobutyric acid),Nle^{27}]hGH-RH(1–28) agmatine (1-amino-4-guanidinobutane) (MZ-5–156), which showed an activity 63–200× higher *in vitro* and 7–16× greater *in vivo* than the standard antagonist (10, 28). This paper reports the synthesis and biological evaluation of a series of GH-RH antagonists with selected hydrophilic or hydrophobic amino acid substitutions. These modifications were aimed to stabilize the helical region

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Abbreviations: Abu, α-aminobutyric acid; Boc, *tert*-butyloxycarbonyl; DCM, dichloromethane; hGH-RH, human growth hormone-releasing hormone; Har, homoarginine; IGF-I, insulin-like growth factor I; Nle, norleucine; PhAc, phenylacetyl; Phe(4-Cl), *para*-chlorophenylalanine; RIA, radioimmunoassay; SCLC, small cell lung carcinoma.

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in the analogs, optimize its amphiphilic secondary structure, and incorporate additional positively charged amino acid residues. The ability of the analogs to inhibit GH-RH-induced GH release was evaluated *in vitro* in a superfused rat pituitary system, as well as *in vivo* after i.v. injection into rats. GH-RH receptor binding affinities of the compounds also were determined.

MATERIALS AND METHODS

Synthesis. GH-RH antagonists were prepared by manual solid-phase peptide synthesis. The amino acid derivatives, resins, and reagents used were obtained from Bachem or Advanced ChemTech. Protected amino acids used in the syntheses were of the L-configuration unless stated otherwise. The α-amino function was protected with the *tert*-butyloxycarbonyl (Boc) group, and the reactive side-chain functional groups were protected as follows: *p*-toluenesulfonyl or nitro for Arg and homoarginine (Har); cyclohexyl for Asp and Glu; benzyloxymethyl for His; 2-chlorobenzyloxycarbonyl for Lys; benzyl for Ser and Thr; and 2-bromobenzyloxycarbonyl for Tyr. The side chains of Asn and Gln were unprotected. All peptides were constructed with an amidated C terminus on *p*-methylbenzhydrylamine resin (0.5–0.8 mmol/g). For the coupling reactions, a 3-fold excess of Boc-amino acid was used with N, N′-diisopropylcarbodiimide as an activating agent in dichloromethane (DCM), dimethylformamide, or mixtures thereof. Boc-Asn and Boc-Gln were coupled as preformed 1-hydroxybenzotriazole esters. After a coupling time of 1 h, the completeness of acylation was monitored at each stage by the standard ninhydrin test. In cases in which incomplete coupling was found, the coupling procedure was repeated, or capping with acetic anhydride in DCM (30% vol/vol) was done before removal of the Boc protecting group. Intermediate deblocking was achieved with 50% (vol/vol) trifluoroacetic acid in DCM, followed by neutralization with 5% (vol/vol) diisopropylethylamine in DCM. After completion of the synthesis and removal of the N-α-Boc protecting group from Tyr^1 or His^1, peptides were acylated with phenylacetic acid (PhAc), indole-3-acetic acid, or 1-naphthylacetic acid by using symmetrical anhydride method. Final deprotection as well as the cleavage of the peptides from the resin was performed with anhydrous hydrogen fluoride in the presence of 10% *m*-cresol at 0°C for 60 min. After removal of the hydrogen fluoride under a stream of nitrogen and *in vacuo*, the free peptides were precipitated with diethyl ether, were filtered and washed with diethyl ether and ethyl acetate, were extracted with 50% (vol/vol) aqueous acetic acid, were diluted with water, and were lyophilized.

Purification and Analysis. Crude peptides were purified on a MacRabbit HPLC system (Rainin Instruments) by using a Vydac (Hesperia, CA) model 218TP5010 reversed-phase column (10 × 250 mm, C_{18} packing with 300-Å pore size and 5-μm particle size). The column was eluted with a solvent system consisting of (*i*) 0.1% (vol/vol) aqueous trifluoroacetic acid and (*ii*) 0.1% trifluoroacetic acid in 70% (vol/vol) aqueous acetonitrile in a linear gradient mode (e.g., 30–55% *ii* in 120 min). The eluent was monitored at 220 nm. The fractions were checked by analytical HPLC, and those with a purity exceeding 95% were pooled and lyophilized. The HPLC analyses of crude and purified peptides were carried out on a Hewlett–Packard model 1090 liquid chromatograph by using a Vydac 218TP52 reversed-phase column (2 × 250 mm, C_{18} packing, 300 Å, 5 μm), with isocratic and/or gradient elution and with the solvent system described above at a flow rate of 0.2 ml/min. The peaks were monitored at 220 and 280 nm. Amino acid analyses of the purified peptides were carried out on a Beckman 6300 amino acid analyzer after hydrolysis of the samples in 6 M hydrochloric acid at 110°C for 24 h in sealed evacuated tubes.

GH-RH Antagonistic Activity *in Vitro*. Antagonistic effect of the analogs on GH-RH-induced GH release was analyzed by using a dispersed rat pituitary superfusion system (29, 30). In brief, after digestion with collagenase, anterior pituitaries from young male Sprague–Dawley rats were dispersed mechanically, were mixed with Sephadex G-10, and were transferred onto superfusion columns by using cells from one pituitary in each column. Medium-199-based tissue culture medium, supplemented with 1 g/liter BSA, 50 mg/liter Penicillin-G, and 87 mg/liter Gentamicin, was perfused through the columns at a rate of 20 ml/h. After an overnight recovery period, 1-ml (3-min) fractions were collected from the effluent media. The cells were exposed periodically to test compounds that were dissolved in fresh medium immediately before application. Unless otherwise indicated, the stimulations were applied with 30-min time intervals and lasted for 3 min. Functional standardization of the system was performed by analyzing GH responses to 50 mM KCl applied in the beginning and at the end of experiment. Between the first and last KCl stimuli, the cells were exposed seven times to 1 nM hGH-RH(1–29)NH_2. The antagonist was applied at 30 or 3 nM concentration for 12 min beginning 9 min before the third GH-RH stimulus and simultaneously with it in the last 3 min. The inhibiting effect of the antagonist at 0, 30, 60, 90, and 120 min after its administration was calculated from the GH responses to the third, fourth, fifth, sixth, and seventh GH-RH stimuli, as compared with the second GH response. After the last GH-RH stimulus, a high concentration (300 nM) of the GH-RH antagonist also was applied for 3 min to assess its intrinsic GH releasing potency. At the end of experiments, the contents of the cells were extracted by passing hypotonic solution (10 mM HCl) through the columns. GH concentrations of the fractions were determined by radioimmunoassay (RIA). RIA results were processed with a special computer program (29). Further statistical analysis was based on the net integral values (area of the response curve above the baseline) of the GH responses.

Receptor Binding. Preparation of rat pituitary membrane fractions and receptor binding of GH-RH were performed as described (31) by using a sensitive *in vitro* ligand competition assay based on binding of ^{125}I-labeled [His^1,Nle^{27}]hGH-RH(1–32)NH_2 to rat anterior pituitary membrane homogenates. In brief, in competitive binding analysis, ^{125}I-labeled [His^1,Nle^{27}]hGH-RH(1–32)NH_2 (0.2 nM) was displaced by GH-RH antagonists at 10^{-6}–10^{-12} M. The final binding affinities were expressed as K_i (dissociation constant of the inhibitor–receptor complex) and were calculated by using by the LIGAND PC computerized curve fitting program of Munson and Rodbard as modified by McPherson (32). Relative affinities compared with [Ac-Tyr^1,D-Arg^2]hGH-RH(1–29)NH_2 (standard antagonist) were calculated as the ratio of K_i of the tested GH-RH antagonists to the K_i of the standard antagonist.

GH-RH Antagonistic Activity *in Vivo*. The potency and duration of antagonistic effect of the analogs were tested *in vivo* on young male Sprague–Dawley rats (200–250 g body weight). The antagonists (80 μg/kg) and hGH-RH(1–29)NH_2 (3 μg/kg) were dissolved in 5.5% sterile mannitol and were given i.v. into the jugular vein of rats under Nembutal anesthesia. In one experiment, five groups of seven animals each were used. The time elapsed between the administration of the antagonist and subsequent GH-RH injection varied between groups (5, 15, 30, and 60 min). Blood samples (0.4 ml) were taken for GH RIA before the administration of the antagonist (measurement of the baseline level is "GH_0") and 5 min after the injection of GH-RH (measurement of the post-stimulus level is "GH_{stimul}"). The controls received mannitol instead of the antagonist, and the GH-RH stimulus was given 5 min later. For statistical evaluation of the serum GH levels, analysis of variance followed by two-tailed Student's *t* test were used.

RIA for GH. Rat GH levels in aliquots of superfusion samples and in serum were measured by double-antibody RIA using materials supplied by the National Hormone and Pituitary Program, Rockville, MD (rat GH-RP-2/AFP-3190B, rat GH-I-6/AFP-5676B, and anti-rat GH-RIA-5/AFP-411S). Interassay variation was <15% and intraassay variation was <10%.

RESULTS

Design and Synthesis. In a search for superactive GH-RH antagonists, 22 analogs of hGH-RH(1–29)NH_2 were prepared by solid-phase peptide synthesis (Table 1). After purification by HPLC, the purity of peptides was examined by analytical HPLC and was found to be >95%. Amino acid analyses of the pure products showed the expected amino acid compositions.

All peptides are based on the common core sequence [PhAc-Tyr^1,D-Arg^2,Phe(4-Cl)^6,Abu^{15},Nle^{27}]hGH-RH(1–29)NH_2, which was responsible for the high biological activity of a previously published antagonist, MZ-5–156 (10) but which contained C-terminal agmatine. In the first 13 peptides with a common D-Arg^{29} C terminus, Arg or norleucine (Nle) substitutions were introduced at various positions of the peptide chain to test their influence on the biological activity. Both Arg and Nle residues favor helical conformation in peptides and can increase amphiphilicity, but Arg is hydrophilic and positively charged whereas, in contrast, Nle is hydrophobic and neutral. Other peptides (**14** to **22**) shared a common D-Arg^{28},Har^{29} C terminus that was expected to confer a strong chemical resistance to enzymatic degradation, given that both D-Arg and Har are nonnatural amino acids. Peptide **14** did not have other substitutions in the core sequence whereas peptides **15** to **22** contained an Arg or Har residue in position 9 combined with other substitutions.

GH-RH Antagonistic Activities *in Vitro*. Inhibitory effects of the analogs on GH-RH-induced GH release in a superfused rat pituitary system are shown in Table 2. Among the first 13 analogs with the same C terminus, peptide **3** (JV-1–10) with Arg^9 substitution had a strong antagonistic potency. This peptide caused an almost total blockade of GH-RH-elicited GH release at 0 min, and it maintained a strong antagonistic effect even 120 min after exposure. Substitution of Arg in other positions yielded moderately potent antagonists in the case of peptides **2** and **8**, with Arg^8 and Arg^{16} replacements, respectively. Arg^7, Arg^{15}, Arg^{18}, or Arg^{19} substitutions were unfavorable, yielding the weak and short-acting antagonists **1**, **6**, **10**, and **12**. Substitution of Nle at different positions proved to be generally unfavorable, yielding weak antagonists or inactive peptides (**5**, **7**, **11**, and **13**). Only Nle^9 substitution (in peptide **4**) provided a strong and long-lasting antagonist whereas peptide **9** with Nle^{16} substitution showed a moderate antagonistic activity. In the pairs of peptides with Arg or Nle substitution at the same position, the Arg-containing analogs showed consistently higher potencies than their Nle-containing counterparts (compare **3** vs. **4**, **6** vs. **7**, **8** vs. **9**, **10** vs. **11**, and **12** vs. **13**).

In the series of peptides containing D-Arg^{28},Har^{29} C terminus (**14** to **22**), peptide **14** (MZ-6–55) without additional substitutions relative to the common core structure exhibited a strong and long-lasting inhibitory potency in the superfusion test. Peptide **15** (JV-1–36), which is the Arg^9-substituted analog of peptide **14**, proved to be an outstandingly good antagonist *in vitro*, causing a total blockade of GH-RH-elicited GH release for at least 90 min under the usual test conditions (30 nM dose). Peptides **19** (with Arg^9,Tyr(Me)^{10} substitution), **20** (JV-1–38) (with Har^9,Tyr(Me)^{10} substitution), and **22** (with 1-naphthylacetyl-His^1, Arg^9 substitution) also showed extremely potent GH-RH antagonistic potencies *in vitro*.

Table 1. Structure of GH-RH antagonists with substitutions in the common core sequence [X-Tyr^1,D-Arg^2,Phe(4-Cl)^6,Abu^{15}, Nle^{27}]hGH-RH(1-29)NH_2*

No.	Code no.	N terminus substitutions, X-	Midchain substitutions	C terminus substitutions
1.	JV-1-12	PhAc-	Arg^7	D-Arg^{29}
2.	JV-1-11	PhAc-	Arg^8	D-Arg^{29}
3.	JV-1-10	PhAc	Arg^9	D-Arg^{29}
4.	JV-1-27	PhAc-	Nle^9	D-Arg^{29}
5.	JV-1-21	PhAc-	Nle^{13}, Nle^{14}	D-Arg^{29}
6.	JV-1-19	PhAc-	Arg^{15}	D-Arg^{29}
7.	JV-1-20	PhAc-	Nle^{15}	D-Arg^{29}
8.	JV-1-17	PhAc-	Arg^{16}	D-Arg^{29}
9.	JV-1-18	PhAc-	Nle^{16}	D-Arg^{29}
10.	JV-1-15	PhAc-	Arg^{18}	D-Arg^{29}
11.	JV-1-16	PhAc-	Nle^{18}	D-Arg^{29}
12.	JV-1-13	PhAc-	Arg^{19}	D-Arg^{29}
13.	JV-1-14	PhAc-	Nle^{19}	D-Arg^{29}
14.	MZ-6-55	PhAc-		D-Arg^{28}, Har^{29}
15.	JV-1-36	PhAc-	Arg^9	D-Arg^{28}, Har^{29}
16.	JV-1-37	Indol-3-acetic acid	Arg^9	D-Arg^{28}, Har^{29}
17.	JV-1-40	1-naphthylacetic acid	Arg^9	D-Arg^{28}, Har^{29}
18.	JV-1-39	PhAc-	Har^9	D-Arg^{28}, Har^{29}
19.	JV-1-41	PhAc-	Arg^9, Tyr(Me)^{10}	D-Arg^{28}, Har^{29}
20.	JV-1-38	PhAc-	Har^9, Tyr(Me)^{10}	D-Arg^{28}, Har^{29}
21.	JV-1-42	PhAc-His^1	Arg^9	D-Arg^{28}, Har^{29}
22.	JV-1-43	1-naphthylacetyl-His^1	Arg^9	D-Arg^{28}, Har^{29}

*X-, N-acyl residue.

Receptor Binding Affinities. Table 3 shows the results of GH-RH receptor binding assays of the most active antagonistic peptides. K_i values of the best antagonists were in the 0.036–0.079 nM range, and the antagonists had 37–82× higher binding affinities than the standard antagonist. The receptor affinities of the peptides were in good agreement with their antagonistic potencies in the *in vitro* superfusion system. Peptide **15** (JV-1–36), which had the strongest *in vitro* inhibitory effect on GH release, was also among the analogs with the best receptor binding affinities, with a K_i value of 0.042 nM. The superiority of Arg substitution relative to Nle is also evident (compare K_i values of peptides **3** vs. **4**, **6** vs. **7**, and **8** vs. **9**) in accord with the *in vitro* superfusion results.

GH-RH Antagonistic Activities *in Vivo*. The antagonists with the highest activity *in vitro* also were evaluated *in vivo* to assess their potencies and duration of action. The results of *in vivo* tests are presented in Table 4. Peptide **15** (JV-1–36), the most potent antagonist *in vitro*, also showed an extremely high and protracted activity *in vivo*, producing a virtually complete blockade of GH-RH-elicited GH release at 5 min after administration, and its action lasted for at least 30 min. Peptides **14** (MZ-6–55) and **20** (JV-1–38) were less potent than JV-1–36 at 5, 15, and 30 min but exhibited a more protracted activity, causing a partial blockade of GH release even 60 min after administration, in contrast to JV-1–36, which was no longer effective at this time. In the same *in vivo* test, the standard antagonist produced only a faint and transient blockade of GH release (28), and the effect of MZ-5–156, a potent GH-RH antagonist developed in our laboratory, lasted solely for ≈15 min (28).

DISCUSSION

In the search for GH-RH antagonists with improved activity, we synthesized and tested biologically a series of hGH-RH analogs. The design approach was based on the introduction in the analogs of helix-prone, hydrophilic, or hydrophobic amino acid substitutions for stabilizing and enhancing the amphiphilic α-helical character of the molecules. It previously was shown that the amphiphilic helical domains of hormones can interact with the amphiphilic biological membrane environment in which the hormone receptors are embedded. Consequently,

Table 2. Inhibitory effects of GH-RH antagonists on the GH-RH-induced GH release in superfused rat pituitary system

Antagonist		Dose, nM	Inhibition of GH release, %					Intrinsic activity*, %
No.	Code no.		0 min	30 min	60 min	90 min	120 min	
Standard antagonist		100	52	13	0	0	0	
1.	JV-1-12	30	59	15	0	0	0	22
2.	JV-1-11	30	69	56	21	18	11	16
3.	JV-1-10	30	91	87	79	72	59	4
4.	JV-1-27	30	68	75	48	52	52	26
5.	JV-1-21	30	10	7	5	0	0	3
6.	JV-1-19	30	64	12	7	0	0	14
7.	JV-1-20	30	39	27	10	9	5	3
8.	JV-1-17	30	75	69	46	24	22	14
9.	JV-1-18	30	52	34	21	10	0	23
10.	JV-1-15	30	70	14	0	0	0	9
11.	JV-1-16	30	0	0	0	0	0	4
12.	JV-1-13	30	29	0	0	0	0	11
13.	JV-1-14	30	30	0	0	0	0	16
14.	MZ-6-55	30	96	89	80	56	42	0
15.	JV-1-36	30	100	100	100	100	94	0
		3	30	47	8	18	6	
16.	JV-1-37	30	100	100	100	100	91	0
17.	JV-1-40	30	79	77	59	59	50	15
		3	25	0	0	0	0	
18.	JV-1-39	30	83	86	80	79	68	13
19.	JV-1-41	30	93	93	97	95	90	7
20.	JV-1-38	30	85	98	91	92	87	15
		3	0	29	0	0	0	
21.	JV-1-42	30	97	91	82	76	65	3
22.	JV-1-43	30	100	100	98	97	90	7
23.	MZ-5-156†	30	95	90	72	65	56	

*At 300 nM concentration relative to the intrinsic activity of 1 nM hGH-RH(1-29)NH_2.
†From ref. 10.

these amphiphilic structures play an important role in the high receptor affinities and biological activities of hormones (33, 34). We selected Nle as a prototypical hydrophobic amino acid substituent and Arg, D-Arg, or Har as prototypical hydrophilic amino acid substituents for our GH-RH antagonists. The basic amino acid arginine was chosen because it was suggested that the positively charged amino acid residues of hormones form salt bridges with the negatively charged phospholipids in biological membranes and are responsible for their high receptor binding affinities (34). Arg was replaced in some cases by D-Arg or Har with the expectation that these nonnatural amino acids might assure a better enzymatic stability for the analogs.

Table 3. K_i values and relative affinities of GH-RH antagonists to membrane receptors on rat anterior pituitary cells

Antagonist		K_i*, nM	Relative affinity†
No.	Code no.		
Standard antagonist		2.96 ± 0.15	1
2.	JV-1-11	0.112 ± 0.01	25
3.	JV-1-10	0.054 ± 0.01	55
4.	JV-1-27	0.124 ± 0.05	24
6.	JV-1-19	0.164 ± 0.06	18
7.	JV-1-20	0.277 ± 0.04	11
8.	JV-1-17	0.104 ± 0.01	28
9.	JV-1-18	0.139 ± 0.02	21
14.	MZ-6-55	0.066 ± 0.02	45
15.	JV-1-36	0.042 ± 0.01	70
16.	JV-1-37	0.044 ± 0.01	67
17.	JV-1-40	0.036 ± 0.01	82
18.	JV-1-39	0.083 ± 0.01	36
19.	JV-1-41	0.044 ± 0.07	67
20.	JV-1-38	0.079 ± 0.01	37
21.	JV-1-42	0.040 ± 0.01	74
22.	JV-1-43	0.038 ± 0.09	78

*Dissociation constant of the inhibitor-receptor complex. Values represent mean ± SEM of two to three independent experiments, each done in duplicate or triplicate.
†Expressed relative to [Ac-Tyr^1,D-Arg^2]hGH-RH(1-29)NH_2 (standard antagonist) = 1.0.

We found that the analog [PhAc-Tyr^1,D-Arg^2,Phe(4-Cl)^6,Arg^9,Abu^{15},Nle^{27},D-Arg^{29}]hGH-RH(1–29)NH_2 (JV-1–10) was a potent antagonist both *in vitro* and *in vivo* with a duration of action of at least 30 min in the endocrine *in vivo* test. [PhAc-Tyr^1,D-Arg^2,Phe(4-Cl)^6,Abu^{15},Nle^{27},D-Arg^{28},Har^{29}]hGH-RH(1–29)NH_2 (MZ-6–55) also had a high and even more protracted antagonistic activity *in vivo* and was capable of producing a partial blockade of GH release 60 min after administration. The long-lasting activity of this peptide could be related to a strong enzymatic resistance of the D-Arg^{28},Har^{29} C terminus, composed of two nonnatural amino acids. Analog[PhAc-Tyr^1,D-Arg^2,Phe(4-Cl)^6,Arg^9,Abu^{15},Nle^{27},D-Arg^{28},Har^{29}]hGH-RH(1–29)NH_2 (JV-1–36), with both an Arg^9 substitution and a D-Arg^{28},Har^{29} C terminus, was the most potent antagonist in the *in vitro* tests, and it also showed a very high *in vivo* activity that lasted for at least 30 min. Replacement of Arg^9 by Har^9 and the introduction of an additional Tyr(Me)^{10} substitution led to a very long-acting antagonist, [PhAc-Tyr^1,D-Arg^2,Phe(4-Cl)^6,Har^9,Tyr(Me)^{10},Abu^{15},Nle^{27},D-Arg^{28},Har^{29}]hGH-RH(1–29)NH_2 (JV-1–38), which was active *in vivo* 60 min after administration. Powerful and protracted *in vivo* activities of these peptides indicate important improvements over earlier antagonists.

Some of hGH-RH antagonists reported herein may find various clinical applications, especially in the treatment of IGF-I- and -II-dependent tumors. It is noteworthy that antagonists MZ-4–71 (9) and MZ-5–156 (10), previously developed

Table 4. *In vivo* inhibitory effects of GH-RH antagonists on the GH release in rats induced by exogenous GH-RH

Antagonist		Serum GH levels in nanograms per milliliter (GH_0* and GH_{stimul}†), GH responses (respGH‡), and relative inhibition (in percent) of GH release§					
No.	Code no.		Control	5 min	15 min	30 min	60 min
3.	JV-1-10	GH_0*	94 ± 24	93 ± 19	75 ± 11	64 ± 5	54 ± 8
		GH_{stimul}†	751 ± 92	200 ± 23	288 ± 55	217 ± 28	386 ± 60
		respGH‡	8.0 ± 0.9	2.8 ± 0.8‖	3.8 ± 0.5‖	3.3 ± 0.3‖	7.3 ± 1.2
		Percent inhibition§	0	75	59	67	10
14.	MZ-6-55	GH_0*	44 ± 10	79 ± 11	53 ± 6	61 ± 21	95 ± 25
		GH_{stimul}†	1013 ± 76	390 ± 80	691 ± 41	738 ± 160	1101 ± 110
		respGH‡	26.2 ± 3.9	5.1 ± 1.1‖	13.6 ± 1.4‖	16.0 ± 4.4‖	16.6 ± 6.0¶
		Percent inhibition§	0	84	50	40	38
15.	JV-1-36	GH_0*	158 ± 24	179 ± 14	139 ± 6	151 ± 9	120 ± 7
		GH_{stimul}†	565 ± 116	198 ± 22	261 ± 24	292 ± 19	479 ± 88
		respGH‡	3.7 ± 0.8	1.1 ± 0.1¶	1.9 ± 0.7	2.0 ± 0.2	3.9 ± 0.7
		Percent inhibition§	0	95	68	64	0
16.	JV-1-37	GH_0*	65 ± 4	180 ± 26	83 ± 8	92 ± 16	88 ± 15
		GH_{stimul}†	351 ± 67	231 ± 39	287 ± 34	278 ± 20	421 ± 88
		respGH‡	5.5 ± 1.0	1.4 ± 0.3‖	3.5 ± 0.1	3.6 ± 0.7	5.4 ± 1.4
		Percent inhibition§	0	91	45	41	2
18.	JV-1-39	GH_0*	61 ± 6	133 ± 24	80 ± 12	77 ± 8	76 ± 4
		GH_{stimul}†	765 ± 89	364 ± 52	555 ± 49	873 ± 162	896 ± 60
		respGH‡	12.8 ± 1.4	3.1 ± 0.6‖	7.7 ± 1.1‖	12.0 ± 2.5	11.9 ± 0.9
		Percent inhibition§	0	82	43	7	8
19.	JV-1-41	GH_0*	47 ± 6	49 ± 10	42 ± 12	55 ± 10	48 ± 13
		GH_{stimul}†	1152 ± 88	565 ± 104	664 ± 143	819 ± 80	776 ± 117
		respGH‡	25.9 ± 3.3	11.5 ± 4.3‖	16.2 ± 6.1	17.9 ± 3.4¶	19.2 ± 5.2
		Percent inhibition§	0	58	41	32	26
20.	JV-1-38	GH_0*	51 ± 5	86 ± 15	66 ± 3	83 ± 13	76 ± 12
		GH_{stimul}†	377 ± 19	245 ± 14	237 ± 52	371 ± 10	387 ± 55
		respGH‡	7.6 ± 0.7	3.1 ± 0.4‖	3.6 ± 0.8‖	4.9 ± 0.9¶	5.4 ± 0.6
		Percent inhibition§	0	68	61	41	34
22.	JV-1-43	GH_0*	66 ± 13	85 ± 10	71 ± 13	92 ± 11	55 ± 6
		GH_{stimul}†	406 ± 111	202 ± 43	245 ± 28	470 ± 42	310 ± 40
		respGH‡	5.8 ± 0.50	2.3 ± 0.3‖	3.6 ± 0.4‖	5.5 ± 0.6	5.8 ± 0.7
		Percent inhibition§	0	74	46	8	1

Results are mean ± SEM. Shown is significant difference from the control group at ¶$P < 0.05$ and at ‖$P < 0.01$ vs. control (Student's two-tailed *t* test).

*GH_0 before treatment with antagonist.

†GH_{stimul} after administration of hGH-RH(1-29)NH_2 to rats pretreated with GH-RH antagonists.

‡$\text{repGH} = (GH_{stimul}/GH_0)$.

§Calculated from GH response values as $100*[1 - (\text{respGH} - 1)/(\text{respGH}_{control} - 1)]$.

in our laboratory, inhibit growth of various human tumors, including osteosarcomas, small-cell and non-small-cell lung carcinomas (SCLC and non-SCLC), prostatic, colorectal, mammary, and renal cancers xenografted into nude mice, as well as Dunning R-3327-AT prostatic cancers in rats and breast tumors in mice (14, 15, 20–24). GH-RH antagonists could inhibit tumor growth through indirect or direct pathways (14). The indirect mechanism would operate through a suppression of GH release from the pituitary and the resulting inhibition of the production of IGF-I in the liver and other tissues. Thus, we have shown that GH-RH antagonists decrease the level of GH and IGF-I in serum of nude mice bearing prostatic and renal cancers, osteosarcomas, and SCLC and non-SCLC xenografts (20–24). However, a major decrease in tumor IGF-I and IGF-II levels found in renal carcinomas, prostate cancers, and non-SCLCs after therapy with GH-RH antagonists (20–24) points to a likely direct effect of GH-RH antagonists on tumors. A strong suppression of IGF-II mRNA expression in DU-145 tumors after treatment with MZ-5–156 supports this view (24). Ongoing oncological studies with the more potent and longer-acting hGH-RH antagonists reported here may help clarify their mechanism of action and establish likely clinical applications.

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