

Lipopeptide antagonists of growth hormone-releasing hormone with improved antitumor activities

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Antagonists of growth hormone-releasing hormone (GHRH) synthesized previously inhibit proliferation of various human cancers, but derivatization with fatty acids could enhance their clinical efficacy. We synthesized a series of antagonists of GHRH(1-29)NH₂ acylated at the N terminus with monocarboxylic or α,ω -dicarboxylic acids containing six to sixteen carbon atoms. These peptides are analogs of prior potent antagonists JV-1-36, JV-1-38, and JV-1-65 with phenylacetyl group at their N terminus. Several new analogs, including MZ-J-7-46 and MZ-J-7-30, more effectively inhibited GHRH-induced GH release *in vitro* in a superfused rat pituitary system than their parent compound JV-1-36 and had increased binding affinities to rat pituitary GHRH receptors, but they showed weaker inhibition of GH release *in vivo* than JV-1-36. All antagonists acylated with fatty acids containing 8–14 carbon atoms inhibited the proliferation of MiaPaCa-2 human pancreatic cancer cells *in vitro* better than JV-1-36 or JV-1-65. GHRH antagonist MZ-J-7-114 (5 μ g/day) significantly suppressed the growth of PC-3 human androgen-independent prostate cancers xenografted into nude mice and reduced serum IGF-I levels, whereas antagonist JV-1-38 had no effect at the dose of 10 μ g/day. GHRH antagonists including MZ-J-7-46 and MZ-J-7-114 acylated with octanoic acid and MZ-J-7-30 and MZ-J-7-110 acylated with 1,12-dodecanedicarboxylic acid represent relevant improvements over earlier antagonists. These and previous results suggest that this class of GHRH antagonists might be effective in the treatment of various cancers.

antagonistic analogs | cancer therapy | proliferation

Antagonistic analogs of human growth hormone-releasing hormone (hGHRH) were designed to inhibit the secretion of GH from the pituitary by blocking the binding of hypothalamic GHRH to GHRH receptors (1–3). The first reported GHRH antagonist, or “standard antagonist,” has the chemical structure of [Ac-Tyr-1, D-Arg-2]hGHRH(1-29)NH₂ (4). GHRH antagonists may find medical use in conditions such as acromegaly, diabetic retinopathy, or diabetic nephropathy (glomerulosclerosis). However, the main applications of GHRH antagonists would be in the field of cancer, in view of their ability to inhibit the proliferation of malignant cells dependent on GHRH or IGF-I and -II (1–3). By suppressing GH secretion from the pituitary GHRH antagonists diminish the synthesis of IGF-I in the liver and other tissues and reduce serum IGF-I levels, thus decreasing growth of IGF-I-dependent tumors (1–3). The proliferation of various experimental tumors can be also inhibited by a direct action of GHRH antagonists on the cancer cells, resulting in the reduction of IGF-I and -II levels in the tumor tissue or their secretion (1–3). Thus, GHRH antagonists previously developed in our laboratory strongly inhibited the growth of various human experimental tumors, including osteosarcomas, small-cell and non-small-cell lung carcinomas (SCLC and non-SCLC), and prostatic, colorectal, mammary, and renal cancers xenografted into nude mice (1–3). The effects were associated with a reduction in the levels of serum IGF-I, or tumoral IGF-I and/or IGF-II (1–3). Moreover, in other cancers,

such as H-69 SCLC or HEC-1A endometrial tumors, in which GHRH itself acts as a growth factor, GHRH antagonists exert their antiproliferative effects by blocking the action of local autocrine/paracrine GHRH, without affecting the IGF system (1–3, 5). Direct inhibitory effects of GHRH antagonists appear to be mediated by the splice variants of GHRH receptors that have been found on various tumors (2, 3, 5, 6).

The therapeutic use of synthetic peptides is limited by their bioavailability. Strategies used to increase drug delivery *in vivo* include the enhancement of stability and circulation time in the bloodstream, targeting of specific tissues or cells, and facilitation of intracytoplasmic delivery (7). Among the modifications of peptides, an increase in lipophilicity by lipidation is a well accepted approach to enhance peptide–membrane interaction. Acylation with fatty acids allows the targeting of proteins and peptides to plasma membranes (8), to various subcellular organelles (9), and also to serum albumin, which has unique ligand binding properties and is abundant in the extracellular fluids (10). Lipopeptides are well defined, can be reproducibly prepared (11), exhibit long-term stability, and lack side effects and inflammatory reactions (12, 13). Unlike many small-peptide drugs, the lipidated peptides have long half-lives (14), probably because they penetrate cell membranes, where they become resistant to clearance and attack by proteases (15). N-terminal acylation of a somatostatin analog with long-chain fatty acids enhanced its stability and antiproliferative activity in human breast adenocarcinoma cells (16). Antagonists of human corticotropin releasing factor lipidated at the N terminus have also been synthesized, and it was found that antagonistic activity was independent of the type of N-terminal acylation (17).

Candidates of GHRH antagonists for clinical development should possess high binding affinities and exert biological effects on both the pituitary and the tumoral splice variant receptors for GHRH. To further elucidate the molecular mode of action of GHRH antagonists and to increase receptor binding affinities and biological activities, we synthesized a new series of GHRH antagonists. These peptides represent analogs of previously synthesized, highly effective GHRH antagonists JV-1-36 and JV-1-65 (1, 18) and are acylated with different monocarboxylic or α,ω -dicarboxylic acids at the N terminus. The length and hydrophobicity of the acylating moieties also were optimized. The new antagonists were then subjected to endocrine and

Conflict of interest statement: Tulane University has applied for a patent on the GHRH antagonists listed in this paper, and M.Z., J.L.V., and A.V.S. are coinventors on that patent.

Abbreviations: GHRH, growth hormone-releasing hormone; SCLC, small-cell lung carcinoma.

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Table 1. Structure of GHRH antagonists acylated at the N-terminus with midchain substitutions in the sequence [X⁰-Tyr¹, D-Arg², Phe(*p*Cl)⁶, Arg⁹, Abu¹⁵, Nle²⁷, D-Arg²⁸, Har²⁹]hGHRH(1–29)NH₂

No.	Code no.	Substitutions at position			
		X ⁰ -	8 Asn	9 Arg	10 Tyr
1	MZ-J-7-50	CH ₃ -(CH ₂) ₄ -CO-			
2	MZ-J-7-52	HOOC-(CH ₂) ₄ -CO-			
3	MZ-J-7-46	CH ₃ -(CH ₂) ₆ -CO-			
4	MZ-J-7-48	HOOC-(CH ₂) ₆ -CO-			
5	MZ-J-7-42	CH ₃ -(CH ₂) ₈ -CO-			
6	MZ-J-7-44	HOOC-(CH ₂) ₈ -CO-			
7	MZ-J-7-34	CH ₃ -(CH ₂) ₁₀ -CO-			
8	MZ-J-7-38	HOOC-(CH ₂) ₁₀ -CO-			
9	MZ-J-7-26	CH ₃ -(CH ₂) ₁₂ -CO-			
10	MZ-J-7-30	HOOC-(CH ₂) ₁₂ -CO-			
11	MZ-J-7-18	CH ₃ -(CH ₂) ₁₄ -CO-			
12	MZ-J-7-22	HOOC-(CH ₂) ₁₄ -CO-			
13	MZ-J-7-54	CH ₃ -(CH ₂) ₁₄ -CO-Phe-			
14	MZ-J-7-56	CH ₃ -(CH ₂) ₁₄ -CO-D-Phe-			
15	MZ-J-7-62	PhAc-Arg-			
16	MZ-J-7-64	PhAc-D-Arg-			
17	MZ-J-7-102	H-			
18	MZ-J-7-112	HOOC-(CH ₂) ₁₂ -CO-	Cit	Cit	
19	MZ-J-7-114	CH ₃ -(CH ₂) ₆ -CO-		Amp	Tyr(Me)
20	MZ-J-7-116	HOOC-(CH ₂) ₈ -CO		Amp	Tyr(Me)
21	MZ-J-7-110	HOOC-(CH ₂) ₁₂ -CO-		Amp	Tyr(Me)
22	JV-1-36*	PhAc		Arg	
23	JV-1-65*	PhAc		Amp	Tyr(Me)
24	JV-1-38*	PhAc		Har	Tyr(Me)

The analogs contain Asn⁸, Arg⁹, and Tyr¹⁰ sequence, except when noted otherwise.

*Reference compounds, reported in refs. 1 and 18.

oncological assays *in vitro* and *in vivo* to characterize their pharmacological properties.

Results

Synthesis. In a search for superactive and long acting GHRH antagonists, 21 analogs of hGHRH(1-29)NH₂ were prepared by solid-phase peptide synthesis and purified by reversed-phase HPLC (Table 1). All peptides contain D-Arg-2, *para*-chlorophenylalanine [Phe(*p*Cl)⁶], α -aminobutyric acid (Abu¹⁵), norleucine (Nle²⁷), D-Arg²⁸, and homoarginine (Har²⁹) substituents, because they are analogs of the previously reported highly potent GHRH antagonists JV-1-36 and JV-1-65 containing these structural features (1,18). Peptides **1** to **16** (Table 1) are derivatives of JV-1-36, which in addition to the above substitutions contains Arg-9 in the molecule (1). Peptides **1**, **3**, **5**, **7**, **9**, **11** are acylated with monocarboxylic acids containing six to sixteen carbon atoms, such as hexanoic, octanoic, decanoic, lauric, myristic, and palmitic acids, whereas the other analogs (**2**, **4**, **6**, **8**, **10**, and **12**) contain α,ω -dicarboxylic acids with six, eight, 10, 12, 14, and 16 carbon atoms, respectively. These latter molecules (**2**, **4**, **6**, **8**, **10**, and **12**) have a negative charge at their N terminus. Analogs **13** and **14** contain an additional Phe and D-Phe, respectively, and are acylated with palmitic acid at the N terminus, whereas, in peptides **15** and **16**, Arg and D-Arg acylated with phenylacetic acid (PhAc) were incorporated at the N terminus. Antagonist **17** has an extra positive charge in the molecule due to the free amino group at the N terminus.

Peptide **18**, which is a derivative of MZ-J-7-78 (18), has citrulline (Cit) 8 and 9 (Cit⁸ and Cit⁹) substituents and, thus, differs in these two positions from JV-1-36 in addition to being acylated with 1,12-dodecanedicarboxylic acid. Compounds

Table 2. Inhibitory effects of GHRH antagonists on the GHRH-induced GH release *in vitro* in superfused rat pituitary cell system

No.	Antagonist Code no.	Inhibition of GH release, %				
		0 min	30 min	60 min	90 min	120 min
Standard antagonist*		52	13	0	0	0
1	MZ-J-7-50	80	87	85	79	69
2	MZ-J-7-52	48	39	44	30	22
3	MZ-J-7-46	95	100	98	92	60
4	MZ-J-7-48	78	80	77	74	73
5	MZ-J-7-42	53	78	88	85	80
6	MZ-J-7-44	85	82	86	76	66
7	MZ-J-7-34	73	69	53	56	60
8	MZ-J-7-38	53	57	34	37	45
9	MZ-J-7-26	40	70	64	65	56
10	MZ-J-7-30	87	84	70	64	65
11	MZ-J-7-18	64	70	65	49	57
12	MZ-J-7-22	13	23	24	29	27
13	MZ-J-7-54	60	32	22	27	39
14	MZ-J-7-56	75	49	32	27	39
15	MZ-J-7-62	79	30	39	24	36
16	MZ-J-7-64	38	36	34	18	26
17	MZ-J-7-102	96	80	63	54	50
18	MZ-J-7-112	58	83	73	56	56
19	MZ-J-7-114	63	65	71	60	53
20	MZ-J-7-116	72	64	46	53	44
21	MZ-J-7-110	76	75	64	48	63
22	JV-1-36*	64	79	75	71	71
23	JV-1-65*	72	85	82	80	77

The antagonists were applied at a 30 nM concentration, except for the standard antagonist, which was applied at 100 nM. Asterisks indicate data taken from ref. 18.

19–21 have *para*-amidino-phenylalanine (Amp) at position 9 and *O*-methyltyrosine [Tyr(Me)] at position 10, and they are derivatives of JV-1-65 (18) acylated with mono- (octanoic acid, in peptide **19**) or dicarboxylic acids (sebacic acid in peptide **20**, and 1,12-dodecanedicarboxylic acid in peptide **21**). Fatty acids applied in peptides **18–21** were chosen on the bases of *in vitro* results obtained with peptides **1–16** (see Tables 2, 3, and 5).

GHRH Antagonistic Activities *in Vitro*. The antagonistic potencies of the analogs *in vitro* were determined by the superfusion assay using rat pituitary cell system. Inhibitory effects of the antagonists on GHRH-induced GH release are shown in Table 2. In the series of analogs of JV-1-36 and JV-1-65 modified with fatty acids at the N terminus, peptides **1**, **3**, **5**, **6**, and **10** showed the highest antagonistic potencies *in vitro*. Their inhibitory activities on GH release exceeded that of the reference peptide JV-1-36. Analog **3** (MZ-J-7-46), which was acylated with octanoic acid, caused a nearly complete blockade of GHRH induced GH release for the first 90 min, being the most potent antagonist in this assay.

Receptor Binding Affinities. Most of the GHRH antagonists modified with fatty acids were also tested in receptor binding experiments on rat pituitary membrane fractions. The results of these experiments are given in Table 3. *K_i* values of the best antagonists (**3**, **10**, **19**, and **21**) were in the 0.061–0.077 nM range and also exceeded the binding affinity of reference peptides JV-1-36 or JV-1-65. Peptide **3** (MZ-J-7-46), which had the strongest inhibitory activity on GH release *in vitro*, also showed the highest GHRH receptor binding affinity, its *K_i* value being 100 times lower than that of the standard antagonist.

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

Antagonist		K_i ,* nM	Relative affinity [†]
No.	Code no.		
Standard antagonist		6.13	1
1	MZ-J-7-50	0.080	77
2	MZ-J-7-52	0.122	50
3	MZ-J-7-46	0.061	100
4	MZ-J-7-48	0.089	69
5	MZ-J-7-42	0.093	66
6	MZ-J-7-44	0.094	65
7	MZ-J-7-34	0.202	30
9	MZ-J-7-26	0.114	54
10	MZ-J-7-30	0.077	80
11	MZ-J-7-18	0.113	54
17	MZ-J-7-102	0.089	69
19	MZ-J-7-114	0.069	89
20	MZ-J-7-116	0.096	64
21	MZ-J-7-110	0.071	86
22	JV-1-36 [‡]	0.091	67
23	JV-1-65 [‡]	0.112	55

*Dissociation constant of the inhibitor–receptor complex. Values were calculated from duplicate or triplicate determinations.

[†]Expressed relative to [Ac-Tyr¹, D-Arg²]hGHRH(1–29)NH₂ (standard antagonist) = 1.0.

[‡]Reference compounds.

GHRH Antagonistic Activities *in Vivo*. Some of the antagonists with high activity *in vitro* were also evaluated *in vivo* to assess their potency and duration of action. The results of *in vivo* tests are presented in Table 4. Peptides **3** (MZ-J-7-46) and **19** (MZ-J-7-114) significantly inhibited the GHRH-evoked GH release *in vivo*. Although the inhibitory activity of these peptides did not reach that of the reference peptide JV-1-36, their effects were nevertheless long lasting. Compound **21** (MZ-J-7-110) showed a significant inhibition only at 5 min.

Cell Proliferation Assay. The inhibitory activities of the analogs, which were effective in superfusion assays, were also tested on the proliferation of MiaPaCa-2 human pancreatic cancer cell line *in vitro* at 10^{-6} , 3×10^{-6} , and 10^{-5} M concentrations (Table 5). Among the peptides tested, analog **5** (MZ-J-7-42) significantly inhibited the cell proliferation even at 10^{-6} M concentration. The proliferation of MiaPaCa-2 human pancreatic cancer cells were significantly inhibited by six peptides (**4**, **5**, **7**, **8**, **10**, and **18**) at 3×10^{-6} M concentration, whereas reference peptides JV-1-36 and JV-1-65 did not show significant inhibition at this concentration. The inhibitory effects of antagonists **4**, **5**, **7**, **10**, and **18** at 3×10^{-6} M concentration were also significantly higher than that of parent peptide JV-1-36. All analogs significantly inhibited cell proliferation at 10^{-5} M concentration. The inhibitory effects of all new antagonists with the exception of peptides **1** and **2** were also significantly higher as compared to that of reference peptide JV-1-36 (Table 5) at 10^{-5} M concentration. The antiproliferative effect of compounds **19** (MZ-J-7-114) and **21** (MZ-J-7-110) also significantly ($P < 0.01$ and $P < 0.001$, respectively) exceeded that of their parent peptide JV-1-65 at 10^{-5} M concentration.

Oncological Study *in Vivo*. The antiproliferative effects of the new antagonist **19** (MZ-J-7-114) and its reference peptide JV-1-38 were investigated on s.c. implanted xenografts of PC-3 human androgen-independent prostate cancer in nude mice. After 4 weeks of treatment, peptide MZ-J-7-114 administered at 5 μ g/day significantly inhibited the growth of PC-3 tumors in nude

mice, and its effect was also significantly greater than that of its parent peptide JV-1-38 at the double dose of 10 μ g/day (Table 6, Fig. 1). The new antagonist significantly inhibited tumor volume by 43% vs. control, whereas JV-1-38 was ineffective. The tumor weight was also significantly lowered in the group treated with MZ-J-7-114 by 58%, whereas antagonist JV-1-38 only caused 10% inhibition (Table 6). Body weights in treated animals did not differ from the control group (data not shown). Serum IGF-I levels were significantly ($P < 0.001$) inhibited by 33% after treatment with antagonist MZ-J-7-114, but not with JV-1-38 (Table 6). Tumoral IGF-II levels were not affected by the antagonists (data not shown).

Discussion

Considerable efforts have been devoted in our laboratory to the synthesis of GHRH antagonists with improved activities (1–3, 18). In this study, a new series of GHRH antagonists was synthesized and modified at the N terminus with fatty acids and tested *in vitro* and *in vivo*. The design of these compounds was based on the incorporation of monocarboxylic and α,ω -dicarboxylic acids into the molecules for the enhancement of peptide-membrane interaction, long-term stability against enzymes, and circulation time of peptides in bloodstream. This approach was previously used successfully for the synthesis of insulin analogs and glucagon-like peptide analogs with protracted activities *in vivo* due to an enhanced binding to serum albumin, which protects the peptides from enzymatic degradation and also reduces their clearance from the blood (10, 19).

Of the 14 analogs of JV-1-36 acylated with fatty acids at the N terminus, two peptides (**4** and **7**) were as active in the superfusion tests *in vitro* as the parent antagonist JV-1-36, and five analogs (**1**, **3**, **5**, **6**, and **10**) were more potent. These latter peptides were acylated at their N terminus with hexanoic, octanoic, decanoic, sebacic, and 1,12-dodecanedicarboxylic acids, respectively. Interestingly, antagonist **17** having free amino group at the N terminus also strongly inhibited GHRH-induced GH release at 0 min, but this effect was of a short duration. The stability of peptide **17** against enzymatic degradation is probably decreased due to its free N terminus. In studies on binding to GHRH receptors on rat pituitary membrane fractions, the relative affinities of analogs **1**, **3**, and **10** were also higher than that of JV-1-36, whereas compounds **5** and **6** showed similar affinities to JV-1-36. Peptide **3** (MZ-J-7-46), having octanoyl group at the N terminus, showed the highest binding affinity among GHRH antagonists, its value being 100 times greater than that of the standard antagonist. The binding affinities of analogs of JV-1-36 acylated with fatty acids were in good correlation with their inhibitory effects in the rat superfusion system.

In addition to the N-acyl analogs of JV-1-36, we synthesized three GHRH antagonists (**19–21**) based on the sequence of JV-1-65, because recent results demonstrated that this antagonist has increased antitumor activity despite a weaker inhibitory effect on the release of GH *in vivo* (2,3,18). Although analogs **19–21** were less active in the rat superfusion assay than JV-1-65, their binding affinities to rat pituitary GHRH receptors exceeded that of reference peptide JV-1-65, indicating the possibility of enhanced peptide–membrane interaction due to the presence of fatty acids at the N termini of these peptides.

Analogues that were effective in the superfusion and receptor binding assay *in vitro* were also tested for the inhibition of GH release *in vivo*. In the series of antagonists (peptides **1**, **3**, **5**, **6**, and **10**), based on the structure of JV-1-36, the acylation caused a decrease in the inhibitory activity *in vivo*. Only the effect of analog **3** (MZ-J-7-46) was significant up to 30 min. However, peptides **19** (MZ-J-7-114) and **21** (MZ-J-7-110) showed increased inhibitory effects compared to their parent peptide JV-1-65. Antagonist **19** (MZ-J-7-114) significantly inhibited GH release *in vivo* even at 60 min after administration. Thus, N-acylation with fatty acids de-

Table 4. Inhibitory effects of GHRH antagonists *in vivo* on the GH release in rats induced by exogenous GHRH

No.	Antagonists Code no.		Serum GH, ng/ml, and relative inhibition				
			Control	5 min	15 min	30 min	60 min
1	MZ-J-7-50	GH _{baseline}	38.0 ± 6.5	37.6 ± 5.3	39.7 ± 11.8	64.6 ± 13.5	50.0 ± 12.7
		GH _{stimulated}	530 ± 101	448 ± 104	497 ± 129	683 ± 36	481 ± 65
		Inhibition, %		17	7	0	10
3	MZ-J-7-46	GH _{baseline}	41.6 ± 11.9	72.9 ± 17.6	11.0 ± 3.9	83.3 ± 25.4	29.5 ± 4.8
		GH _{stimulated}	1,215 ± 177	901 ± 181	715 ± 112*	768 ± 60*	945 ± 172
		Inhibition, %		28	41	40	23
5	MZ-J-7-42	GH _{baseline}	56.9 ± 14.4	50.6 ± 10.8	30.6 ± 11.4	68.4 ± 26.7	48.7 ± 13.0
		GH _{stimulated}	933 ± 350	1,210 ± 224	747 ± 134	1,186 ± 118	982 ± 96
		Inhibition, %		0	21	0	0
6	MZ-J-7-44	GH _{baseline}	50.4 ± 8.2	38.1 ± 19.2	39.7 ± 10.4	31.0 ± 6.2	37.4 ± 8.6
		GH _{stimulated}	1,390 ± 96	1,008 ± 225	1,368 ± 129	1,280 ± 204	1,253 ± 141
		Inhibition, %		28	2	8	10
10	MZ-J-7-30	GH _{baseline}	26.1 ± 10.9	16.4 ± 4.1	19.4 ± 5.3	29.5 ± 9.4	16.6 ± 3.0
		GH _{stimulated}	850 ± 159	863 ± 109	861 ± 77	812 ± 160	917 ± 106
		Inhibition, %		0	0	4	0
19	MZ-J-7-114	GH _{baseline}	48.7 ± 3.6	18.5 ± 4.4	33.5 ± 12.3	33.7 ± 7.8	25.0 ± 7.2
		GH _{stimulated}	1,379 ± 162	867 ± 116*	788 ± 114*	912 ± 88	809 ± 107*
		Inhibition, %		38	44	35	42
21	MZ-J-7-110	GH _{baseline}	57.0 ± 10.8	26.7 ± 5.8	22.0 ± 10.9	49.2 ± 6.3	24.9 ± 4.7
		GH _{stimulated}	1,197 ± 131	707 ± 61†	927 ± 204	1,100 ± 110	906 ± 55
		Inhibition, %		42	23	8	25
22	JV-1-36 [‡] (Exp. 1)	GH _{baseline}	158 ± 24	179 ± 14	139 ± 6	151 ± 9	120 ± 7
		GH _{stimulated}	565 ± 116	198 ± 22¶	261 ± 24†	292 ± 19†	479 ± 88
		Inhibition, %		95	71	66	19
22	JV-1-36 [‡] (Exp. 2)	GH _{baseline}	48.0 ± 11.0	39.2 ± 12.2	73.1 ± 9.39	66.1 ± 9.67	73.2 ± 14.5
		GH _{stimulated}	1,307 ± 94.9	180 ± 28.1¶	689 ± 69.3¶	1,126 ± 101	1,337 ± 154
		Inhibition, %		89	50	15	0
23	JV-1-65 [‡] (Exp. 1)	GH _{baseline}	56.5 ± 11.0	38.6 ± 5.86	112 ± 17.0	78.5 ± 32.1	ND
		GH _{stimulated}	906 ± 86.5	601 ± 64.2	919 ± 112	920 ± 123	ND
		Inhibition, %		35	0	0	ND
23	JV-1-65 [‡] (Exp. 2)	GH _{baseline}	109 ± 20.2	79.0 ± 19.7	82.7 ± 24.4	ND	ND
		GH _{stimulated}	1,819 ± 191	1,551 ± 291	1,590 ± 264	ND	ND
		Inhibition, %		15	13		

GH levels are mean ± SEM of five to seven animals per group. *, *P* < 0.05 vs. control; †, *P* < 0.01 vs. control; ‡, reference compounds; ¶, *P* < 0.001 vs. control; ND, not determined.

creased the activity *in vivo* of analogs of JV-1-36, but enhanced the antagonistic effect of analogs of JV-1-65. The reason for the different effect of N-acylation with fatty acids in the two series of peptides is not known. In previous work, it was also found that inhibitory effects on GH release *in vivo* did not always parallel the activities *in vitro* (18).

Because the main applications of GHRH antagonists would be in the area of oncology, we tested the new analogs for inhibition of cancer cell proliferation *in vitro*. MiaPaCa-2 human pancreatic cancer line was chosen for the initial oncological evaluation of GHRH antagonists, because this cell line is a particularly appropriate model for testing the direct effects of GHRH antagonists. MiaPaCa-2 cells express the tumoral splice variants of GHRH receptors, whereas other receptors related to GHRH such as those for vasoactive intestinal peptide, pituitary adenylate cyclase activating peptide, secretin, or glucagon, that could interfere in the proliferation assays, are not present (6, 20). Our results indicate that all antagonistic analogs of GHRH acylated with mono- or dicarboxylic acids containing 8–14 carbon atoms have increased antiproliferative effect *in vitro* as compared to their parent peptides JV-1-36 and JV-1-65. However, analogs **1** and **2**, acylated with the shorter hexanoic or adipic acids containing six carbon atoms, were less potent.

Some of the peptides described in this paper have been and continue to be tested in various tumor models *in vivo* to more completely characterize oncological activities. Thus, in the

present study, we found that GHRH antagonist 19 (MZ-J-7-114) given at 5 µg/day significantly inhibited the growth of PC-3 human androgen-independent prostate cancers xenografted into nude mice and reduced serum IGF-I levels, whereas antagonist JV-1-38 had no effect at the dose of 10 µg/day. Our group also demonstrated that MZ-J-7-114 at 10 µg doses very strongly inhibits the growth of H460 and A549 human non-SCLC tumors in nude mice (2, 3). In another study, in which nude mice bearing H-69 human SCLC tumors were treated with GHRH antagonists JV-1-65 and MZ-J-7-110 at doses of 10 µg/day, it was found that both antagonists significantly decreased tumor volume and weight, the inhibitory effect of MZ-J-7-110 being greater than that of the parent peptide JV-1-65 (21).

In the course of synthesis of previous series of GHRH antagonists we began to observe a dissociation between the endocrine and oncological activities. Among the older GHRH antagonists, JV-1-36 and JV-1-38 inhibited the growth of PC-3 and other human cancers xenografted into nude mice at a dose of 20 µg/day, and newer and more potent antagonist JV-1-65 was effective at 10 µg/day (1–3, 18, 21). Antagonist JV-1-36 strongly inhibited GH release in rats, whereas antagonists JV-1-38 and JV-1-65 had only weak and nonsignificant inhibitory effects on GH release *in vivo* (18). Although the new analogs MZ-J-7-114 and MZ-J-7-110, acylated with fatty acids, show a modest enhancement of inhibitory actions on GH release *in vivo* as compared to their parent peptides JV-1-65 and JV-1-38, their

Table 5. Inhibitory effects of GHRH antagonists on the growth of MiaPaCa-2 human pancreatic carcinoma cell line *in vitro*

GHRH antagonist no.	GHRH antagonist code no.	T/C % on MiaPaCa-2 cell line at the dose of											
		Significance vs. 10 ⁻⁶ M			Significance vs. 3 × 10 ⁻⁶ M			Significance vs. 10 ⁻⁵ M			Significance vs. Control		
		Control	JV-1-36	JV-1-65	Control	JV-1-36	JV-1-65	Control	JV-1-36	JV-1-65	Control	JV-1-36	JV-1-65
Experiment 1													
	Control				100.0 ± 1.43								
22	JV-1-36*	103.92 ± 2.26	NS		100.00 ± 2.26	NS					79.32 ± 0.83	<0.001	
3	MZ-J-7-46	94.83 ± 3.35	NS	NS	93.09 ± 2.79	NS	NS				33.82 ± 0.98	<0.001	<0.001
4	MZ-J-7-48	96.44 ± 1.95	NS	NS	90.86 ± 1.19	<0.05	<0.05				28.87 ± 0.91	<0.001	<0.001
6	MZ-J-7-44	95.67 ± 1.67	NS	NS	92.25 ± 1.46	NS	NS				36.54 ± 0.63	<0.001	<0.001
10	MZ-J-7-30	93.20 ± 1.43	NS	<0.01	72.07 ± 1.66	<0.001	<0.001				19.24 ± 0.98	<0.001	<0.001
Experiment 2													
	Control				100.00 ± 0.90								
22	JV-1-36*	97.36 ± 1.46	NS		98.82 ± 1.17	NS					82.04 ± 1.22	<0.001	
1	MZ-J-7-50	98.15 ± 1.39	NS	NS	95.99 ± 1.64	NS	NS				82.70 ± 2.82	<0.001	NS
2	MZ-J-7-52	97.12 ± 1.54	NS	NS	96.10 ± 1.28	NS	NS				87.58 ± 1.80	<0.001	NS
5	MZ-J-7-42	91.75 ± 0.70	<0.01	NS	90.50 ± 1.60	<0.001	<0.01				40.72 ± 1.15	<0.001	<0.001
3	MZ-J-7-46	95.40 ± 0.95	NS	NS	92.95 ± 1.90	NS	NS				48.32 ± 1.10	<0.001	<0.001
7	MZ-J-7-34	94.68 ± 1.37	NS	NS	90.68 ± 1.27	<0.001	<0.01				37.65 ± 0.59	<0.001	<0.001
8	MZ-J-7-38	95.30 ± 0.75	NS	NS	92.55 ± 0.85	<0.05	NS				48.02 ± 0.60	<0.001	<0.001
9	MZ-J-7-26	97.31 ± 0.98	NS	NS	97.12 ± 1.61	NS	NS				37.70 ± 0.63	<0.001	<0.001
18	MZ-J-7-112	96.25 ± 0.56	NS	NS	89.63 ± 1.13	<0.001	<0.001				43.66 ± 0.87	<0.001	<0.001
Experiment 3													
	Control				100.00 ± 1.31								
22	JV-1-36*	96.99 ± 2.10	NS	NS	92.08 ± 1.39	NS	NS	NS			61.7 ± 1.69	<0.001	NS
23	JV-1-65*	98.17 ± 2.40	NS	NS	99.41 ± 1.89	NS	NS	NS			62.4 ± 1.69	<0.001	NS
21	MZ-J-7-110	97.49 ± 1.71	NS	NS	97.01 ± 1.68	NS	NS	NS			42.04 ± 1.82	<0.001	<0.001
19	MZ-J-7-114	98.32 ± 2.25	NS	NS	93.35 ± 2.25	NS	NS	NS			51.30 ± 1.00	<0.001	<0.01

*Reference compounds.

endocrine activity on the pituitary *in vivo* is weaker than that of JV-1-36 or of other antagonists with outstanding inhibitory effects on GH release, such as JV-1-63 (18). However, the oncological activity of the new antagonists is substantially increased, as demonstrated by the significant inhibitory effect of MZ-J-7-114 and MZ-J-7-110 in small dose on the growth of PC-3 and other tumors as cited above. This finding indicates an augmentation of the direct effects on tumors, mediated by tumoral GHRH receptors.

In conclusion, our results demonstrate the beneficial effect of modification at the N terminus with octanoic or 1,12-dodecanedicarboxylic acids on the inhibitory activity of GHRH antagonists. The enhanced antiproliferative activities of GHRH antagonists acylated with fatty acids at their N terminus indicate marked improvements over earlier antagonists. Some of the analogs of this type could be considered for clinical development for treatment of various cancers.

Materials and Methods

Peptide Synthesis. GHRH antagonists with amidated C termini were prepared by solid-phase methodology on *p*-methylbenzhydrylamine resin using Boc-chemistry, as described (18). All

amino acid derivatives, resins, and reagents used were obtained from Bachem (Torrance, CA), RSP Amino Acids DBA (Shirley, MA), or Sigma. N-terminal acylation of peptides was performed after completion of the synthesis and removal of the N- α -Boc protecting group from Tyr-1. Resin-bound peptides were acylated with mono- or α,ω -dicarboxylic acids using 10 equivalents of preformed anhydrides of the appropriate fatty acids. Final deprotection and cleavage of the peptides from the resin with anhydrous hydrogen fluoride, as well as their purification and analysis by semipreparative and analytical HPLC, were done as described (18).

Evaluation of GHRH Antagonistic Activity *in Vitro*. Antagonistic activities of the peptides on GH release were determined by using a superfused rat pituitary cell system as described (18). Briefly, the antagonists were perfused through the cells at 30 nM concentration, followed by stimuli of 1 nM hGHRH(1-29)NH₂ applied 0, 30, 60, 90, and 120 min later. The amount of GH secreted by the cells was determined by RIA. The inhibitory activities of antagonists were expressed as % inhibition of the reference GH response induced by GHRH in the absence of antagonist.

Table 6. Effects of GHRH antagonists MZ-J-7-114 and JV-1-38 on the growth of human PC-3 androgen-independent prostate cancer in nude mice and serum IGF-levels

Treatment	Tumor volume, mm ³		Tumor volume doubling time, days	Tumor weight, mg (% inhibition)	Serum IGF-I, ng/ml (% inhibition)
	Initial	Final (% inhibition)			
Control	28.4 ± 4.2	351.1 ± 84.9	8.3 ± 0.7	282.7 ± 72.1	262 ± 18.1
JV-1-38* (10 μ g/day)	29.3 ± 3.8	351.0 ± 72.9	8.7 ± 0.7	254.3 ± 52.0 (10)	293 ± 28.0
MZ-J-7-114 (5 μ g/day)	26.5 ± 3.7	199.3 ± 40.4 (43) ^{††}	12.6 ± 2.5 [†]	119.1 ± 25.3 (58) ^{††}	175 ± 12.9 (33%) ^{¶¶}

*Reference compound; †, *P* < 0.05 vs. control; ‡, *P* < 0.05 vs. JV-1-38; ¶, *P* < 0.001 vs. control and JV-1-38.

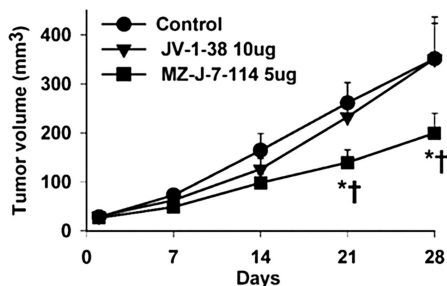


Fig. 1. Inhibitory effects of GHRH antagonists on the tumor growth of PC-3 human androgen-independent prostate cancers xenografted into nude mice. *, $P < 0.05$ vs. control; †, $P < 0.05$ vs. JV-1-38.

Receptor Binding. Preparation of rat pituitary membrane fractions and receptor binding of GHRH were performed as previously described (18, 22), using a sensitive *in vitro* ligand competition assay based on binding of ^{125}I -labeled $[\text{His}^1, \text{Nle}^{27}]\text{hGHRH}(1-32)\text{NH}_2$ to rat anterior pituitary membrane homogenates.

GHRH Antagonistic Activity *in Vivo*. The inhibitory effect of antagonists (80 $\mu\text{g}/\text{kg}$ i.v.) on the GH release induced by hGHRH(1-29) NH_2 (3 $\mu\text{g}/\text{kg}$ i.v.) in rats was tested as described (18). Separate groups (five to seven rats per group) received the GHRH stimulus at varying time intervals (5, 15, 30, or 60 min) after the antagonist. Serum GH levels before the administration of the antagonist ($\text{GH}_{\text{baseline}}$) and 5 min after the injection of GHRH ($\text{GH}_{\text{stimulated}}$) were measured by RIA. Controls received no antagonist before the GHRH stimulus. Percent inhibition of GH release was calculated as described (18).

All experiments *in vivo* were performed in accordance with institutional ethical guidelines for the care and use of experimental animals.

Cell Proliferation Assay. MiaPaCa-2 human pancreatic cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture as described (20). For evaluation of

the inhibitory activity of the analogs on the proliferation of this cell line *in vitro*, MiaPaCa-2 cells were seeded into 96-well plates and exposed to 10^{-6} , 3×10^{-6} , and 10^{-5} M concentrations of the antagonists, in octuplicate wells each, for 70–72 h. At the end of treatment, cell proliferation was measured by the tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay as described (20).

Oncological Study *in Vivo*. PC-3 human androgen-independent prostate cancer cell line was obtained from the American Type Culture Collection. Male athymic (Ncr *nu/nu*) nude mice, ≈ 6 weeks old on arrival, were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). S.c. xenografts of PC-3 tumor tissue were performed as described (23). The animals (8–10 mice per group) were then treated s.c. with GHRH antagonists JV-1-38 (10 $\mu\text{g}/\text{day}$) and MZ-J-7-114 (5 $\mu\text{g}/\text{day}$). Tumors were evaluated after 4 weeks of treatment, and serum IGF-I and tumoral IGF-II were also measured as described (23).

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 $<10\%$.

Statistical Analysis. Data are expressed as means \pm SEM. Statistical evaluation of the results was done by Student's two-tailed *t* test or one-way ANOVA followed by Bonferroni's test.

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