Int. J. Peptide Protein Res. **36**, 1990, 499–505

Synthesis and *in vitro* and *in vivo* activity of analogs of growth hormone-releasing hormone (GH-RH) with *C*-terminal agmatine

MARTA ZARANDI, VALER CSERNUS, LAZARO BOKSER, SANDOR BAJUSZ, KATE GROOT, and ANDREW V. SCHALLY

Endocrine, Polypeptide and Cancer Institute, Veterans Administration Medical Center and Section of Experimental Medicine, Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana, USA

Received 11 January, accepted for publication 19 May 1990

In the search for more active analogs of human growth hormone-releasing hormone (GH-RH), 37 new compounds were synthesized by solid phase methodology, purified, and tested biologically. Most of the analogs contained a sequence of 27 amino acids and *N*-terminal desaminotyrosine (Dat) and *C*-terminal agmatine (Agm), which are not amino acids. In addition to Dat in position 1 and Agm in position 29, the majority of the analogs had Ala^{15} and Nle^{27} substitutions and one or more additional L- or D-amino acid modifications. [Dat^{1}, Ala^{15}, Nle^{27}]GH-RH(1-28)Agm (MZ-2-51) was the most active analog. Its *in vitro* GH-releasing potency was 10.5 times higher than that of GH-RH(1-29)NH_2 and in the i.v. *in vivo* assay, MZ-2-51 was 4–5 times more active than the standard. After s.c. administration to rats, MZ-2-51 showed an activity 34 times higher at 15 min and 179 times greater at 30 min than GH-RH(1-29)NH_2 and also displayed a prolonged activity. D-Tyr^{10}, D-Lys^{12}, and D-Lys^{21} homologs of MZ-2-51 also showed enhanced activities. Thus, [Dat^{1}, D-Tyr^{10}, Ala^{15}, Nle^{27}]GH-RH(1-28)Agm (MZ-2-159), [Dat^{1}, D-Lys^{12}, Ala^{15}, Nle^{27}]GH-RH(1-28)AGM (MZ-2-57), and [Dat^{1}, Ala^{15}, D-Lys^{21}, Nle^{27}]GH-RH(1-28)Agm (MZ-2-75) were 4-6 times more active *in vitro* than GH-RH(1-29)NH_2. *In vivo*, after i.v. administration, analog MZ-2-75 was equipotent and analogs MZ-2-159 and MZ-2-57 about twice as potent as the standard. After s.c. administration, the potencies of MZ-2-57 and MZ-2-75 were 10–14 times higher than the standard at 15 min and 45–89 times greater when determined at 30 min. Most of the analogs containing two or more D-amino acid substitutions were less active than GH-RH(1–29)NH_2 or inactive. Our studies indicate that very potent GH-RH analogs can result from the combination of agmatine in position 29 with other substitutions.

Key words: human growth hormone-releasing hormone; GH-RH analogs

Growth hormone-releasing hormone (GH-RH) has been isolated from human pancreatic tumors and characterized as amidated 44-amino acid peptide hGH-RH(1–44)NH_2 (1) and the free acid of the first 40 amino acid residues hGH-RH(1–40)OH (2). These GH-RH sequences are identical to those present in human hypothalamus (3). Porcine and bovine hypothalamic GH-RH share an extensive structural homology with hGH-RH(1–44) (4). Structure activity studies have demonstrated the importance of the NH_2-terminus and showed that GH-RH(1–29)NH_2 possesses the full intrinsic activity *in vitro* and is the shortest fragment endowed with this property (5,6). A number of potent analogs of GH-RH(1–29)NH_2 have been synthesized (7–13) in order to enhance its biological activity. Among a series of GH-RH(1–29)NH_2 analogs with a single D-amino acid substitution, the D-Ala^{2}, D-Asp^{3}, D-Asn^{8}, D-Tyr^{10}, D-Asp^{25}, D-Met^{27}, D-Ser^{28}, and D-Arg^{29} derivatives were as potent *in vitro* as hGH-RH(1–29)NH_2 (10). Substitutions with the corresponding D-isomer in position 1, 2, 3, or 8 also increased the *in vivo* GH releasing activity (7–9).

Abbreviations of the amino acids are in accord with the recommendations of the IUPAC-IUB JCBN (*J. Biol. Chem.* **264**, 668–673, 1989). Additional abbreviations: Ac_2O, acetic anhydride; Agm, agmatine = 4-guanidino-butylamine; Boc, *tert.*-butyloxycarbonyl; Dat, desaminotyrosine (3-(4-hydroxyphenyl)propionyl-); DCM, dichloromethane; DIC, *N,N'*-diisopropylcarbodiimide; DMF, dimethylformamide; GH, growth hormone; GH-RH, growth hormone-releasing hormone; hGH-RH, human GH-RH; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; i.m., intramuscular; i.v., intravenous; MeCN, acetonitrile; RP, reversed-phase; s.c., subcutaneous; TEA, triethylamine; TFA, trifluoroacetic acid.

Replacement of Tyr[1] by desNH$_2$-Tyr[1] led to analogs with increased biological activities as a result of enhanced resistance of the *NH*$_2$-terminus to enzymatic degradation (12,13). *In vitro* activity also increased when Gly[15] was replaced by Ala[15] (12–14). A few multiple replacement analogs and cyclic analogs also showed increased effects (12,13). Since agmatine (Agm) can effectively substitute the Arg residue in certain enzyme inhibiting peptides (15), we have previously synthesized GH-RH analogs with Agm in position 29 and shown that they are very potent (16). Based on these earlier findings, we have synthesized a series of multiple replacement analogs of hGH-RH(1–29)NH$_2$ containing Agm in position 29 in an attempt to further increase the biological activity. This paper describes our findings.

EXPERIMENTAL PROCEDURES

Peptide synthesis

The various GH-RH analogs were synthesized by solid-phase methodology (17) using Beckman model 990 peptide synthesizer or manual solid-phase peptide synthesis equipment. For peptides with an amidated *COOH*-terminus, *p*-methylbenzhydrylamine resin (0.68 mmol/g) was used. For analogs with *C*-terminal Agm, aminomethyl resin (0.47 mmol/g) was employed as starting material.

Protected amino acids used in the synthesis were of the L-configuration unless stated otherwise. The α-amino function was protected exclusively with the Boc group. The reactive side chain functional groups were protected as follows: benzyl for threonine and serine; 2,6-dichlorobenzyl for tyrosine; *p*-toluene-sulfonyl for arginine; 2-chlorobenzyloxycarbonyl for lysine; and cyclohexyl for aspartic acid and glutamic acid. The side chains of asparagine and glutamine were unprotected. All protected amino acids and resins were purchased from Bachem Inc., Torrance, CA, USA. All solvents were reagent or HPLC grade.

For the synthesis of Agm-peptides, the guanidino group of Agm was linked to the aminomethyl resin through the sulfophenoxyacetyl group (18), which permits intermediate deprotection with TFA and final deprotection with HF. Briefly, crystalline 4-chlorosulfonyl phenoxyacetic acid, obtained from phenoxyacetic acid and chlorosulfonic acid in chloroform, was reacted with Boc-Agm in aqueous acetone at pH 11–12 to yield Boc-N^G-[4-(carboxymethoxy)benzene-sulfonyl]agmatine as a thick oil, which was coupled to an aminomethyl resin with DIC/HOBt in DMF, then the Boc group was removed with 50% TFA in dichloromethane (18). The protected amino acids were coupled to the growing peptide chain in 3 M excess in the presence of diisopropylcarbodiimide for 60–120 min. Boc-Asn and Boc-Gln were coupled with 1-hydroxybenzotriazole ester. Coupling steps were monitored by the standard ninhydrin test (19) and repeated when necessary with preformed symmetrical anhydrides in DMF–DCM (1:1).

The Boc group was removed after each completed coupling step by two treatments with 50% TFA in dichloromethane for 5 and 25 min each. Deblocking steps were followed by neutralization with 10% TEA in dichloromethane. Acetylation of the *N*-terminus was performed twice with 30% acetic anhydride in dichloromethane first for 10 and then for 20 min. The standard cycle of coupling, acetylation of unreacted amino groups, and deblocking is shown in Table 1.

After completion of the synthesis, dried and protected peptide-resin was treated with a mixture consisting of 1.0 mL m-cresol and 10 mL anhydrous hydrogen fluoride per gram of peptide-resin for 45 min at 0° to cleave the peptide from the resin as well as to deprotect the side chain functional groups. After the removal of the hydrogen fluroide, under a stream of nitrogen and vacuum, the free peptides were precipitated with ether, filtered, washed with ether and ethyl acetate, extracted with 50% acetic acid, diluted with water, and lyophilized.

Purification

The purification of the crude peptides was performed on a MacRabbit HPLC system (Rainin Instrument Co. Inc., Woburn, MA) (with a Knauer UV Photometer and a Kipp and Zonen BD40 Recorder) using a 2.14 × 250 mm DYNAMEX column packed with spherical C18 silica gel (300 Å pore size, 12 μm particle size) (Rainin Inc.). The column was eluted with a solvent system consisting of (A) 0.1% aqueous TFA and (B) 0.1% TFA in 70% aqueous MeCN in a linear gradient mode (e.g. 30–70% B in 80 min). The eluent was monitored at 220 nm and fractions were examined by analytical HPLC using a Hewlett-Packard Model HP-1090 liquid chromatograph. Analyses were carried out on a W-Porex C18 reversed-phase column (4.6 × 250 mm, 5 μm particle size, 300 Å pore size) (Phenomenex, Rancho Palos Verdes, CA) using gradient elution (A: 0.1% aqueous TFA; B: 0.1% aqueous TFA in 70% acetonitrile) with a flow rate of 1.2 mL/min. The peaks were continuously monitored at 220 and 280 nm. Fractions were pooled for maximum purity rather than yield.

The purified peptides also were characterized by amino acid analysis after hydrolysis in 6 M hydrochloric acid (110°, 20 h) in tubes sealed under vacuum. Amino acid analyses were carried out on a Beckman 6300 amino acid analyzer.

Bioassays

The GH-releasing effect of the peptides was tested *in vitro* using a system based on superfusion of male rat anterior pituitary cells (20). The net integral values of

TABLE 1

Step	Reagents and Operations	Mixing times (min)
1	Deprotection: 50% TFA in DCM	5 + 25
2	2-propanol wash	2
3	DCM wash	2
4	Neutralization: 10% TEA in DCM	2
5	2-propanol wash	2
6	Neutralization: 10% TEA in DCM	3
7	MeOH wash	2
8	DCM wash (3 times)	3
9	Coupling: Boc-protected amino acid (3 equiv.) in DCM or DMF plus DIC (3 equiv.), or preformed HOBt ester of the BOC-protected amino acid in DMF (3 equiv.)	60 – 120
10	MeOH wash (3 times)	2
11	DCM wash (3 times)	2
12	Ac_2O in DCM (30% solution)	10 + 20
13	MeOH wash (3 times)	2
14	DCM wash (3 times)	2

the GH release were evaluated. Analogs very active *in vitro* were also tested *in vivo* in pentobarbital-anesthetized adult male rats using i.v., s.c., and i.m. routes of administration (21). In both *in vitro* and *in vivo* experiments, hGH-RH(1–29)NH_2 was used as a standard. The responses induced by the analogs were compared at two dose levels to those produced by the standard. GH released in *in vitro* and *in vivo* experiments was measured by RIA using materials supplied by the National Hormone and Pituitary Program, Baltimore, Maryland. Inter-assay variation was less than 15% and intra-assay variation was 10% or less. The potencies of the peptides resulting from 4-point assays were calculated by the factorial analysis of Bliss and Marks with 95% confidence limits.

RESULTS AND DISCUSSION

After a series of hGH-RH(1–29)NH_2 analogs were synthesized by solid-phase methodology and purified by preparative RP-HPLC, the purity of the peptides was found to be greater than 95% as determined by analytical HPLC. Amino acid analyses were consistent with expected values (± 5%). The peptides were then tested *in vitro* for their ability to release GH from dispersed rat pituitary cells, as compared to GH-RH(1–29)NH_2. Table 2 presents the structures and relative *in vitro* GH-releasing potencies of the 33 new hGH-RH(1–29)NH_2 analogs. All the analogs contained Ala^{15}, Nle^{27}, and Agm^{29} and most of them had desaminotyrosine in position 1. In order to avoid oxidative inactivation of the analogs, Met^{27} was replaced by Nle in every analog. The replacement of Met^{27} by Nle decreases the activity of some analogs. Thus [D-Ala^2, Nle^{27}]GH-RH(1–29)NH_2 had only one-third of the activity of the corresponding Met^{27}-peptide when it was administered s.c. (9). However, our analog, [Dat^1, Ala^{15}, Nle^{27}]GH-RH(1–28)Agm (MZ-2-51) showed a potency 10.5 times greater *in vitro* than hGH-RH(1–29)NH_2. Since the GH releasing potencies were retained or moderately decreased by a single substitution with the corresponding D-amino acid at position 8, 10, 12, 21, or 25 (10), we synthesized homologs of MZ-2-51 containing one or more D-amino acid substitutions in these positions. Among these homolog peptides, containing one D-amino acid in the molecule, only the D-Lys^{12} (MZ-2-57), D-Lys^{21} (MZ-2-75), and D-Tyr^{10} (MZ-2-159) analogs had increased potencies, which were 4.1, 4.0, and 5.7 times higher than the standard, respectively *in vitro*. Homologs containing D-Ala^{10} (MZ-2-189) and D-Glu^{25} (MZ-2-221) showed a moderate decrease in potency. All analogs containing two or more D-amino acids in the molecule displayed decreased activities, with the exception of MZ-2-87, MZ-2-177, and MZ-2-229. [Dat^1, D-Tyr^{10}, D-Lys^{12}, Ala^{15}, Nle^{27}]GH-RH(1–28)Agm (MZ-2-177) had 2.1 times greater activity, and [Dat^1, D-$Lys^{12,21}$, Ala^{15}, Nle^{27}]GH-RH(1–28)Agm-(MZ-2-87) and [Dat^1, D-Tyr^{10}, Ala^{15}, D-Glu^{25}, Nle^{27}]GH-RH(1–28)Agm (MZ-2-229) showed no significant change in potency *in vitro*, compared to GH-RH(1-29)NH_2. D-Ala^{10} homologs of MZ-2-51 and MZ-2-87, i.e. MZ-2-189 and MZ-2-193, exhibited a reduction in the potencies to 60% and 81%, respectively of the standard. These results are in accord with previous findings for single D-Ala^{10} substitution in GH-RH(1-29)NH_2 (10). However, if we compare the potencies of these analogs with that of MZ-2-51, the incorporation of D-Ala in position 10 decreased the GH-releasing activity to one-sixth.

TABLE 2

In vitro *GH-releasing activities of GH-RH(1-28)Agm analogs as compared to hGH-RH(1-29)NH_2 in the superfused rat pituitary cell system*

No.	Code No.	Peptides residues at positions												Relative potency[a]
		1	2	8	10	12	15	21	22	23	25	27	29	
	hGH-RH(1-29)NH_2	Tyr	Ala	Asn	Tyr	Lys	Gly	Lys	Leu	Leu	Asp	Met	ArgN-H_2	1.0
1	MZ-2-51	Dat					Ala					Nle	Agm	10.5
2	MZ-2-57	Dat				D-Lys	Ala					Nle	Agm	4.07
3	MZ-2-75	Dat					Ala	D-Lys				Nle	Agm	4.0
4	MZ-2-159	Dat			D-Tyr		Ala					Nle	Agm	5.7
5	MZ-2-189	Dat			D-Ala		Ala					Nle	Agm	6.6
6	MZ-2-221	Dat					Ala					Nle	Agm	1.3
7	MZ-2-87	Dat				DLys	Ala	DLys				Nle	Agm	2.1
8	MZ-2-117	Dat			DTyr	D-Lys	Ala					Nle	Agm	2.1
9	MZ-2-229	Dat			DTyr		Ala				D-Glu	Nle	Agm	0.9
10	MZ-2-193	Dat			D-Ala	D-Lys	Ala	D-Lys				Nle	Agm	0.81
11	MZ-2-199	Dat			D-Tyr	Ala	Ala					Nle	Agm	0.15
12	MZ-2-203	Dat		D-Ser		Ala	Ala					Nle	Agm	0.23
13	MZ-2-207	Ac-Tyr	D-Ala			Ala	Ala					Nle	Agm	0.7
14	MZ-2-205	Dat		D-Asn		Ala	Ala					Nle	Agm	0
15	MZ-2-165	Dat				D-Lys	Ala		Ala			Nle	Agm	4.7
16	MZ-2-171	Dat					Ala	D-Lys	Ala			Nle	Agm	3.1
17	MZ-2-153	Dat				D-Lys	Ala	D-Lys	Ala			Nle	Agm	0.8
18	MZ-2-233	Dat			D-Tyr		Ala		Ala		D-Glu	Nle	Agm	0.52
19	MZ-2-111	Ac-Tyr	D-Ala			D-Lys	Ala	D-Lys				Nle	Agm	0.1
20	MZ-2-209	Dat		D-Asn		D-Lys	Ala	D-Lys				Nle	Agm	0.02
21	MZ-2-213	Dat		D-Ser			Ala	D-Lys				Nle	Agm	0.33
22	MZ-2-217	Dat		D-Ser		D-Lys	Ala					Nle	Agm	0.46
23	MZ-2-223	Dat		D-Ser			Ala				D-Glu	Nle	Agm	0.21
24	MZ-2-237	Ac-Tyr	D-Ala	D-Ser			Ala				D-Glu	Nle	Agm	0.5
25	MZ-2-211	Dat		D-Asn		D-Lys	Ala					Nle	Agm	0.4
26	MZ-2-227	Dat		D-Asn		D-Lys	Ala				D-Glu	Nle	Agm	0.05
27	MZ-2-235	Dat		D-Asn			Ala				D-Glu	Nle	Agm	0.1
28	MZ-2-113	Ac-Tyr	D-Ala				Ala	D-Lys				Nle	Agm	0.02
29	MZ-2-81	Dat				D-Lys	Ala			D-Leu		Nle	Agm	0.4
30	MZ-2-85	Dat			D-Tyr	D-Lys	Ala			D-Leu		Nle	Agm	0.1
31	MZ-2-93	Dat				D-Lys	Ala		D-Leu			Nle	Agm	0.6
32	MZ-2-225	Dat				D-Lys	Ala				D-Glu	Nle	Agm	0
33	MZ-3-43	Dat			D-Tyr		Ala	D-Lys				Nle	Agm	0.7

[a]Expressed relative to GH-RH(1-29)NH_2 = 1.0.

Since single Ala substitution in position 22 was reported to double the relative potency (14), we synthesized some peptides containing Ala in position 22, in addition to other modifications and also incorporated Ala^{12} into several related analogs. All Ala^{12} analogs (MZ-2-199, MZ-2-203, and MZ-2-207) were less active than the standard, but only one (MZ-2-205) was completely inactive. In contrast to Ala^{12} substitutions, Ala^{22} homologs of MZ-2-57 and MZ-2-75, i.e. MZ-2-165 and MZ-2-171, showed increased *in vitro* activities which were 4.7 and 3.1 times higher than the standard. However, compared with the parent peptides MZ-2-57 and MZ-2-75, the analogs MZ-2-165 and MZ-2-171 with Ala^{22} substitutions displayed the same or slightly reduced GH releasing activities. Analogs MZ-2-153 and MZ-2-233 were the Ala^{22} homologs of MZ-2-87 and MZ-2-229, respectively, and evoked only 80% and 52% of the response to the standard. The activity of analog MZ-2-233 with Ala^{22} was also diminished as compared with parent peptide MZ-2-229.

Although most of the analogs including MZ-2-57, MZ-2-177, and MZ-2-165 which contained Lys^{12} or Lys^{21} in the D-configuration showed enhanced GH release in the superfused system, the peptides where both Lys were D-isomers (MZ-2-87, MZ-2-111, MZ-2-153, MZ-2-193, and MZ-2-209) were less active than the analogs with only one D-Lys.

Replacement of Asn^8 by D-Ser^8 was reported to enhance the GH-releasing potency of [D-Ala^2]GH-RH(1-29)NH_2 (22). Contrary to our expectations, the D-Ser^8 analogs containing agmatine and other modifications (MZ-2-203, MZ-2-213, MZ-2-217, MZ-2-223, and MZ-2-237) were less active *in vitro* than the standard. Although the GH-releasing potency of the parent peptide is retained after single D-Asn^8 substitution (10), our multiple replacement analogs containing D-Asn^8 showed significantly decreased activities (MZ-2-209, MZ-2-211, MZ-2-227, and MZ-2-235) and one of them (MZ-2-205) was inactive.

Analogs MZ-2-111 and MZ-2-113 represented D-Ala^2 homologs of MZ-2-87 and MZ-2-75, and were both almost completely inactive in the superfusion system. These results indicate that incorporation of D-Ala^2 in combination with Ac-Tyr^1 in our peptide containing D-Lys^{12}, Ala^{15}, D-Lys^{21}, Nle^{27}, Agm^{29} is not favorable for the biological activity.

Table 3 illustrates the relative *in vitro* potencies of MZ-2-51, MZ-2-57, and their Arg^{29}-NH_2 and D-Arg^{29}-NH_2 homologs MZ-2-117, MZ-2-123, MZ-2-135, and MZ-2-139. The relative potencies of the peptides increase in D-Arg < Arg < Agm order for analogs with substitutions of the Dat^1, Ala^{15}, and Nle^{27} type contained in MZ-2-51. Similarly the activity of Agm analog MZ-2-57 was higher than that of corresponding peptides MZ-2-123 and MZ-2-135 with Dat^1, D-Lys^{12}, Ala^{15}, and Nle^{27} with L- or D-Arg^{29}, the order of potency of analogs being Arg < D-Arg < Agm. These observed potency orders indicate that the substitution of Agm at the *C*-terminus can convey the highest activity in both cases. A two-point contact with the *C*-terminal binding sites of the receptor through the amino and the guanidino group of agmatine in our analogs may be more favorable than a three-point contact in the case of GH-RH(1-29)NH_2.

The activities *in vivo*, resulting from i.v. administration of MZ-2-51, MZ-2-57, MZ-2-75, MZ-2-87, MZ-2-159, and MZ-2-165 as compared with GH-RH(1-29)NH_2 are summarized in Table 4. Analog MZ-2-51 produced significantly higher GH release at the same dose than the other analogs tested. MZ-2-51 was 4.4 times more active than the standard when GH release was measured at 5 min and 5.6 times more potent at 15 min. MZ-2-51 was 2.5–3 times more potent than its D-Lys^{12} homolog (MZ-2-57), and about 3–4 times more active than its D-Lys^{21} homolog (MZ-2-75). Considering the GH levels produced 5 and 15 min after intravenous administrations, MZ-2-51 was also 3–4 times more potent than its D-$Lys^{12,21}$ homolog (MZ-2-87) and more than twice as active as analogs MZ-2-159 and MZ-2-165.

Table 5 represents the subcutaneous activities of some GH-RH(1-28)Agm analogs. Analog MZ-2-51 was again the most active. Its GH-releasing activity

TABLE 3

In vitro *GH-releasing potencies of some hGH-RH(1-29)NH_2 analogs containing Agm,* L-*Arg, or* D-*Arg at the* C-*terminus*

No.	Code No.	Peptide residues at positions					Relative potency[a]
		1	12	15	27	29	
	hGH-RH(1-29)NH_2	Tyr	Lys	Gly	Met	Arg-NH_2	1.0
1	MZ-2-51	Dat		Ala	Nle	Agm	10.5
2	MZ-2-57	Dat	D-Lys	Ala	Nle	Agm	3.8
34	MZ-2-117	Dat		Ala	Nle	Arg-NH_2	8.5
35	MZ-2-123	Dat	D-Lys	Ala	Nle	Arg-NH_2	1.8
36	MZ-2-139	Dat		Ala	Nle	D-Arg-NH_2	2.36
37	MZ-2-135	Dat	D-Lys	Ala	Nle	D-Arg-NH_2	2.6

[a]Expressed relative to GH-RH(1-29)NH_2 = 1.0.

TABLE 4

In vivo *GH-releasing activities of hGH-RH(1-28)Agm analogs after intravenous administration to rats, as compared to hGH-RH(1-29)NH$_2$*

No.	Code No.	Peptide residues at positions 1	10	12	15	21	22	27	29	Relative potency[a] 5 min	15 min
	hGH-RH(1-29)NH$_2$	Tyr	Tyr	Lys	Gly	Lys	Leu	Met	Arg-NH$_2$	1.0	1.0
1	MZ-2-51	Dat			Ala			Nle	Agm	4.42	5.57
2	MZ-2-57	Dat		D-Lys	Ala			Nle	Agm	1.94	1.85
3	MZ-2-75	Dat			Ala	D-Lys		Nle	Agm	1.07	1.91
4	MZ-2-159	Dat	D-Tyr		Ala			Nle	Agm	1.9	2.47
7	MZ-2-87	Dat		D-Lys	Ala	D-Lys		Nle	Agm	1.03	1.83
15	MZ-2-165	Dat		D-Lys	Ala		Ala	Nle	Agm	1.9	2.05

[a]Expressed relative to GH-RH(1-29)NH$_2$ = 1.0.

TABLE 5

In vivo *GH-releasing activities of hGH-RH(1-28)Agm analogs after subcutaneous administration to rats as compared to hGH-RH(1-29)NH$_2$*

No.	Code No.	Peptide residues at positions 1	12	15	21	27	29	Relative potency[a] 15 min	30 min
	hGH-RH(1-29)NH$_2$	Tyr	Lys	Gly	Lys	Met	ArgNH$_2$	1.0	1.0
1	MZ-2-51	Dat		Ala		Nle	Agm	34.35	179.1
2	MZ-2-57	Dat	D-Lys	Ala		Nle	Agm	14.30	88.96
3	MZ-2-75	Dat		Ala	D-Lys	Nle	Agm	10.5	45.0

[a]Expressed relative to GH-RH(1-29)NH$_2$ = 1.0.

was 34 times higher after 15 min and 179 times greater than the standard after 30 min. MZ-2-51 was about twice as active as its D-Lys^{12} homolog (MZ-2-57) and 3–4 times more potent than its D-Lys^{21} homolog (MZ-2-75). On intramuscular administration, MZ-2-51 was also more potent than other analogs (21). These results will be reported elsewhere (21).

Our results demonstrate that replacement of Arg^{29} by agmatine in the GH-RH(1-29)NH$_2$ molecule appears to convey resistance to enzymatic degradation at the *C*-terminus of the analogs. This effect is similar to that produced by D-Ala^2 (7) or desaminotyrosine (12, 16) substitutions which protect the *N*-terminus of GH-RH(1-29)NH$_2$ against aminopeptidases. The favorable effect of Agm^{29} is also supported by our findings that [Dat^1, Ala^{15}, Nle^{27}]GH-RH(1-28)Agm (MZ-2-51) exerts a longer action than the corresponding Arg^{29}-NH$_2$ peptide (MZ-2-117) when injected i.m. (21). The structure of our most active analog MZ-2-51 is very close to the natural sequence, since it has only two amino acid substitutions (Ala^{15} and Nle^{27}) in the molecule and contains only 27 amino acids inasmuch as desaminotyrosine and agmatine are not amino acids. Other analogs containing Agm similarly showed enhanced GH-releasing activities both *in vitro* and *in vivo* in rats. Our Agm analog also displayed increased activity as compared to GH-RH(1-29) NH$_2$ when tested in cattle (23). Because of their high activity, some of our Agm analogs of GH-RH(1-29)NH$_2$, particularly MZ-2-51, may find clinical and veterinary applications.

ACKNOWLEDGMENTS

We thank Dr. Robert J. Kupper, W.R. Grace and Co.-Conn., for a gift of Boc-agmatine resin used in the synthesis of analogs; Dr. Alan H. Deutch of W.R. Grace and Co.-Conn. for helpful discussions; and Mr. Donald Olsen for the amino acid analyses. We also thank Dr. S. Song for performing the hormone assays and the National Hormone and Pituitary Program (NHPP) for the gifts of materials used in radioimmunossays. The work described in this paper was supported by a grant from W.R. Grace and Co.-Conn., (to A.V.S.), and by the Medical Research Service of the Veterans Administration.

REFERENCES

1. Guillemin, R., Brazeau, P., Bohlen, P., Esch, F., Ling, N. & Wehrenberg, W.B. (1982) *Science* **218,** 585–587
2. Rivier, J., Spiess, J., Thorner, M. & Vale, W. (1982) *Nature* **300**, 276–278
3. Bohlen, P., Brazeau, P., Bloch, B., Ling, N., Gaillard, R. & Guillemin, R. (1983) *Biochem. Biophys. Res. Commun.* **114,** 930–936
4. Guillemin, R., Zeytin, F., Ling, N., Bohlen, P., Esch, F., Brazeau, P., Bloch, B. & Wehrenberg, W.B. (1984) *Proc. Soc. Exp. Biol. Med.* **175,** 407–413
5. Wehrenberg, W.B. & Ling, N. (1983) *Biochem. Biophys. Res. Commun.* **115,** 525–530
6. Ling, N., Baird, A., Wehrenberg, W.B., Ueno, N., Munegumi,

T. & Brazeau, P. (1984) *Biochem. Biochem. Biophys. Res. Commun.* **123,** 854–861
7. Lance, V.A., Murphy, W.A., Sueiras-Diaz, J. & Coy, D.H. (1984) *Biochem. Biophys. Res. Commun.* **119,** 265–272
8. Coy, D.H., Murphy, W.A., Sueiras-Diaz, J., Coy, E.J. & Lance, V.A. (1985) *J. Med. Chem.* **28,** 181–185
9. Coy, D.H., Murphy, W.A., Lance, V.A. & Heiman, M.L. (1986) *Peptides* **7,** 49–52
10. Sato, K., Hotta, M., Kageyama, J., Chiang, T.-Ch., Hu, H.-Y., Dong, M.-H. & Ling, N. (1987) *Biochem. Biophys. Res. Commun.* **149,** 531–537
11. Felix, A.M., Heimer, E.P., Mowles, T.F., Eisenbeis, H., Leung, P., Lambros, T.J., Ahmad, M., Wang, C.-T. & Brazeau, P. (1986) in *Peptides 1986* (Theodoropoulos, D., ed.), pp. 481–484, Walter de Gruyter, Berlin
12. Felix, A.M., Wang, C.-T., Heimer, E., Fournier, A., Bolin, D.R., Ahmad, M., Lambros, T., Mowles, T. & Miller, L. (1988) in *Peptides: Chemistry and Biology* (Marshall, G.R., ed.), pp. 465–467, Escom, Leiden
13. Felix, A.M., Heimer, E.P., Wang, C.-T., Lambros, T.J., Fournier, A., Mowles, T.F., Maines, S., Campbell, R.M., Wegrzynski, B.B., Toome, V., Fry, D. & Madison, V.S. (1988) *Int. J. Peptide Protein Res.* **32,** 441–454
14. Ling, N., Baird, A., Wehrenberg, W.B., Munegumi, T. & Ueno, N. (1985) in Proceedings of the "Quo Vadis?" Symposium, Sanofi Group, May 29–30, 1985, Toulouse-Labege, France, pp. 309–322
15. Bajusz, S., Barabas, E. & Bagdy, D. (1983) in *Peptides 1982* (Blaha, K. & Malon, P., eds.), pp. 643–647, Walter de Gruyter, Berlin
16. Kovacs, M., Gulyas, J., Bajusz, S. & Schally A.V. (1988) *Life Sci.* **42,** 27–35
17. Merrifield, R.B. (1963) *J. Am. Chem. Soc.* **85,** 2149–2154
18. Gulyas, J., Bajusz, S. & Schally A.V. (1988) in *Peptides:* Proc. of the 50th Anniversary Symp. of the Nobel-Prize of A. Szent-Gyorgyi (Penke, B. & Torok, A., eds.), pp. 113–116, Walter de Gruyter, Berlin
19. Kaiser, E., Colescott, R.L., Bossinger, C.D. & Cook, P.I. (1970) *Anal. Biochem.* **34,** 595–598
20. Csernus, V.J. & Schally, A.V. (1989) in *Neuroendocrine Research Methods: The Dispersed Cell Superfusion System* (Greenstein, B.D., ed.), in press
21. Bokser, L., Zarandi, M. & Schally A.V. (1990) *Life Sci.* **46,** 999–1005
22. Sawano, S., Ohashi, S., Masuda, H. & Ozaki, S. (1988) in The Endocrine Society 70th Annual Meeting: Program and Abstract, pp. 86 (Abstract No. 264), New Orleans, LA
23. Roberge, S., Johnson, H.E., Zarandi, M. & Schally, A.V. (1989) *J. Animal Sci.* **40,** 150–153

Address:

Dr. Andrew V. Schally (151)
Veterans Administration Medical Center
1601 Perdido Street
New Orleans, LA 70146
Phone: (504) 589-5230
Fax: (504) 566-1625