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Potent agonists of growth hormone-releasing hormone

Part I

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Analogs of the 29 amino acid sequence of growth hormone-releasing hormone (GH-RH) with agmatine (Agm) in position 29 have been synthesized by the solid phase method, purified, and tested *in vitro* and *in vivo*. The majority of the analogs contained desaminotyrosine (Dat) in position 1, but a few of them had Tyr^1, or N-$MeTyr^1$. Some peptides contained one or more additional L- or D-amino acid substitutions in positions 2, 12, 15, 21, 27, and/or 28. Compared to the natural sequence of GH-RH(1-29)NH_2, [Dat^1,Ala^{15}]GH-RH(1-28)Agm (MZ-3-191) and [D-Ala^2,Ala^{15}]GH-RH(1-28)Agm (MZ-3-201) were 8.2 and 7.1 times more potent *in vitro*, respectively. These two peptides contained Met^{27}. Their Nle^{27} analogs, [Dat^1,Ala^{15},Nle^{27}]GH-RH(1-28)Agm (MZ-2-51), prepared previously (9), and [D-Ala^2,Ala^{15},Nle^{28}]GH-RH(1-28)Agm (MZ-3-195) showed relative *in vitro* potencies of 10.5 and 2.4, respectively. These data indicate that replacement of Met^{27} by Nle^{27} enhanced the GH-releasing activity of the analog when the molecule contained Dat^1-Ala^2 residues at the *N*-terminus, but peptides containing Tyr^1-D-Ala^2 in addition to Nle^{27} showed decreased potencies. Replacement of Ser^{28} with Asp in multi-substituted analogs of GH-RH(1-28)Agm resulted in a decrease in *in vitro* potencies compared to the parent compound. Thus, the Ser^{28}-containing MZ-2-51, and [Dat^1,Ala^{15},D-Lys^{21},Nle^{27}]GH-RH(1-28)Agm, its Asp^{28} homolog (MZ-3-149), possessed relative activities of 10.5 and 5.6, respectively. *In vivo* after the iv injection, the analogs [Dat^1,Ala^{15},Nle^{27},Asp^{28}]GH-RH(1-28)Agm (MZ-3-149), [Dat^1, Ala^{15}]GH-RH(1-28)Agm, (MZ-3-191) and [D-Ala^2,Ala^{15}]GH-RH(1-28)Agm (MZ-3-201) showed a potency equivalent to 7.6, 4.9 and 3.3 times that of GH-RH(1-29)NH_2, respectively, at 5 min and 20.3, 4.3 and 1.7 times higher, respectively, at 15 min. After sc administration, analogs MZ-3-149, MZ-3-191, and MZ-3-201 were shown to be 63.7, 55.2 and 56.8 times more potent than the parent hormone at 15 min and 57.6, 60.6, and 42.6 times more active, respectively, at 30 min. In addition, MZ-3-149 had prolonged GH-releasing activity as compared to the standard, and proved to be more potent than MZ-2-51, the most active member of our previous series (8, 9). Our studies indicate that very potent GH-RH analogs can result from the combination of agmatine in position 29 with other substitutions.

Key words: growth hormone-releasing hormone; GH-RH analogs; solid phase peptide synthesis; structure-activity relationships

Growth hormone-releasing hormone (GH-RH) exists in different molecular forms, such as GH-RH(1-44)NH_2 (1) and GH-RH(1-40)OH (2). Structure-activity studies have demonstrated that the amino-terminal region is required for the GH-releasing activity (2, 3). It has been observed that replacement of Tyr^1 by desNH_2-

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Abbreviations of the amino acids are in accordance with the recommendations of the IUPAC-IUB JCBN (*J. Biol. Chem.* **264**, 668–673; *European J. Biochem.* **138**, 11–37, 1984). Additional abbreviations: Ac_2O, acetic anhydride; Agm, agmatine = 4-guanidino-butylamine; Boc, *tert.*-butyloxycarbonyl-; Dat, desaminotyrosine; DCM, dichloromethane; Cl-SPA, 4-chlorophenysulfonyl phenoxyacetic acid, DIC, *N,N'*-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; GH, growth hormone; GH-RH, growth hormone-releasing hormone; hGH-RH, human GH-RH; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MeCN, acetonitrile; im, intramuscular; iv, intravenous; sc, subcutaneous; TFA, trifluoroacetic acid.

Tyr^1 (Dat) led to analogs with increased biological activities as a result of enhanced resistance of the NH_2-terminus to enzymatic degradation (4, 5, 6–9), and D-amino acid substitutions in positions 1, 2, or 3 resulted in analogs with increased GH-releasing activities both *in vitro* and *in vivo* (10–12).

Studies with deletion analogs of the carboxy-terminus have showed that GH-RH(1-29)NH_2 possesses the full intrinsic activity *in vitro* (10, 13) and *in vivo* (14) and is the shortest fragment endowed with this property (3, 13). Since agmatine (Agm), an amino formed by decarboxylation of arginine, i.e. 4-guanidinobutylamine, with the following structure: H_2N-$(CH_2)_4$-NH-$C(NH_2)$=NH, can be effectively substituted for the Arg residue in certain enzyme-inhibiting peptides (15), we have previously synthesized GH-RH analogs with Agm in position 29, i.e. the analogs of GH-RH(1-28)Agm, and shown that some of them are very potent (6–9) *in vitro* and *in vivo*.

The *in vitro* activities are increased in some analogs containing Gly^{15} replaced by Ala^{15} or other hydrophobic residues (4, 5, 16). In order to avoid oxidative inactivation of the analogs, Met^{27} was replaced by Nle in some peptides. Replacement of Met^{27} by Nle decreases the activity of some analogs (17), but in few multiple replacement analogs and also in GH-RH(1-29)NH_2 increased activities were found (16, 17). All of our earlier synthesized analogs that displayed enhanced GH-releasing activities contained Nle^{27} (6–9).

The aim of our work was to synthesize analogs of GH-RH(1-28)Agm with high GH-releasing effect. We compared the activities of analogs containing Met^{27} with those of analogs containing Nle^{27} and investigated how some changes in the amino- or carboxy-terminal region of the molecules influence the GH-releasing potencies. This paper describes our findings.

MATERIALS AND METHODS

Amino acid derivatives

All protected amino acids and resins used in peptide synthesis were purchased from Bachem Inc., Torrance, CA. 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 Thr and Ser; 2,6-dichlorobenzyl for Tyr and *N*-MetTyr; *p*-toluenesulfonyl for Arg, 2-chlorobenzyl-oxycarbonyl for Lys, cyclohexyl for Asp, and sulfoxide for Met. The side-chains of asparagine and glutamine were unprotected.

Chemicals (DMF, Ac_2O, TFA, DIC, and DIEA) used in peptide synthesis were obtained from Aldrich Co., Milwaukee, WI, and were analytical or HPLC grade. HOBt was obtained from Advanced ChemTech, Louisville, KY, and HF was obtained from Matheson.

Synthesis of Boc-agmatine

20 g (86 mmol) agmatine sulfate was suspended in 300 mL 50% aqueous acetone, 25 mL (179 mmol) triethylamine and 25 g (115 mmol) di-*tert.*-butyl dicarbonate was added in small portions with stirring. After 2 h, an additional 12.5 g di-*tert.*-butyl dicarbonate was added, followed by another 12.5 g portion. After overnight stirring, the insoluble part was removed by filtration. The basic solution (pH = 10–11) was extracted with diethyl ether (3 × 100 mL) and lyophilized. The residue was dissolved in 200 mL of distilled water and 15 g sodium bicarbonate was added. The solution was kept at 4° in the refrigerator until white solid began to form. The yield of Boc-agmatine hydrogen carbonate was 15.8 g (67%), melting point 128–130°, with decomposition. The crude material can be recrystallized from water.

Synthesis of 4-chlorosulfonyl phenoxyacetic acid (Cl-SPA)

8 g (54 mmol) phenoxyacetic acid was dissolved in 75 mL chloroform, cooled to 0° and 16.5 mL chlorosulfonic acid in 25 mL chloroform was added dropwise. After 25 min, the temperature was allowed to reach 25°. The mixture was then poured on ice and the aqueous phase was extracted twice with 50 mL ether. The combined organic phase was dried over anhydrous sodium sulfate and evaporated *in vacuo*. The solid was recrystallized from ether-hexane (1:1 mixture). The yield was 11.5 g (87.5%). The melting point was 159–162°.

The purity was checked on Sigma F254 TLC analytical plates in solvent A-ethyl acetate-acetic acid 9:1 (v/v) and solvent B-ether-hexane 2:1 (v/v) plus one drop of acetic acid. Rf^1: Phenoxyacetic acid in system A = 0.85 and in system B = 0.34. Rf_2: Chlorosulfonyl phenoxyacetic acid in system A = 0.77 and in system B = 0.17.

Synthesis of Boc-agmatine-N^G-sulfonyl-phenoxyacetic acid (Boc-Agm-SPA)

Boc-agmatine hydrogen carbonate 4.5 g (11 mmol) was taken up in 50 mL aqueous acetone and the pH was adjusted to 12 with NaOH. 4-Chlorosulfonyl phenoxyacetic acid 5 g (20 mmol) in 15 mL acetone was added in small portions with continuous stirring and cooling in an ice bath and the pH was kept at 12 with 4 M NaOH. The reaction was followed by analytical RP-HPLC. The reaction was stopped after 3 h by acidifying the solution with 10% citric acid.

The acetone was removed *in vacuo* and the residue was extracted with 3 × 100 mL ethyl acetate. The combined organic phase was successively washed with saturated NaCl solution and then dried over sodium sulfate. After evaporation to dryness at room temperature, the crude Boc-Agm-SPA was obtained as an oil containing Agm-SPA and 4-sulfonyl phenoxyacetic acid in various proportions. After HPLC purification, the crys-

talline material formed has a melting point of 146–154°. The yield was 2.8 g (71%).

Coupling of the Boc-Agm-SPA to the solid support
1.5 g (3.5 mmol) Boc-Agm-SPA was dissolved in 20 mL DMF and reacted with 0.94 g (7 mmol) HOBt in the presence of 0.55 mL (3.5 mmol) *N,N'*-diisopropylcarbodiimide at room temperature for 30 min. The resulting activated ester solution was added to 2.5 g neutralized and DMF washed aminomethyl resin (0.6 mmol free amino group/gram resin). The resin was stirred for 90 min and the unreacted free amino functions were blocked with 5% acetylimidazole in DMF and washed several times with DMF and methanol.

Peptide synthesis
The analogs of GH-RH(1-29)NH_2 were prepared by manual solid-phase synthesis using the method described by Kent (18) on an aminomethyl resin (0.47 mmol/g, Bachem Inc., Torrance, CA) for analogs with *C*-terminal Agm.

The protected amino acids were coupled in 3 M excess in the presence of *N,N'*-diisopropylcarbodiimide for 20–40 min. Boc-Asn-OH and Boc-Gln-OH were coupled via their 1-hydroxybenzotriazolyl ester, and Boc-Arg(Tos)-OH was coupled in the presence of 1 equiv. HOBt. Coupling steps were monitored by the standard ninhydrin test (19) and repeated when necessary with preformed symmetrical anhydrides. When the repeated coupling was incomplete, acetylation was performed with an excess (30-fold) of acetic anhydride in DMF.

The Boc group was removed after each completed coupling step by two treatments with 100% TFA for 1 and 4 min each. Deblocking steps were followed by washing with DMF and neutralization with 5% DIEA in DMF.

After completion of the synthesis, the 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 and to deprotect the side chain functional groups. After the removal of the hydrogen fluoride under a stream of nitrogen *in vacuo*, the free peptides were precipitated with ether, filtered, washed with ether and ethyl acetate, extracted with 50% acetic acid, diluted with water, and lyophilized. Methionine sulfoxide was reduced to methionine by treatment with thioglycolic acid at 57° overnight and the solutions of the peptides were lyophilized.

Purification
Crude peptides were purified by using a MacRabbit HPLC system (Rainin Instrument Co. Inc., Woburn, MA) equipped with a Knauer UV Photometer and a Kipp and Zonen BD40 Recorder and a 21.2 × 250 mm DYNAMAX 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). Fractions were pooled for maximum purity rather than yield.

Analytical HPLC
The analyses of crude and purified peptides were carried out on a Hewlett-Packard Model HP-1090 liquid chromatography using a 4.6 × 250 mm W-Porex C-18 silica gel column (particle size 5 μm, pore size 300 Å, Phenomenex, Rancho Palos Verdes, CA). Elution was 1.2 mL/min with solvent system described above (linear gradient mode).

Amino acid analysis
Analyses were performed with a Beckman 6300 amino acid analyzer. Samples were hydrolyzed at 110° for 20 h in evacuated tubes containing 6 M hydrochloric acid.

Bioassays
The GH-releasing activities of the peptides were assayed *in vitro* using a system based on superfusion of male rat anterior pituitary cells (20). Each peptide was perfused through the cells for 3 min at various concentrations (30 pM–1 μM). GH content of the 1 mL fractions collected was determined by RIA. The net integral values of the GH release were evaluated and related to that of 1 nM GH-RH(1-29)NH_2.

For *in vivo* experiments, male Sprague-Dawley rats weighing 230–280 g were anesthetized with 60 mg/kg of sodium pentobarbital (Nembutal, Abbott laboratories, North Chicago, IL) between 0800 h and 0900 h and 20 min later the analogs MZ-3-149, MZ-3-191, MZ-3-201 and GH-RH(1-29)NH_2, dissolved in 0.9% saline, were injected iv or sc; control groups were injected only with saline. Blood samples were drawn from the jugular vein before and 5, 15, 30 and occasionally 60 min after the administration of the analogs and were replaced with saline. Groups of 5–7 rats were used in each experiment. GH levels were measured by RIA using materials supplied by the National Hormone and Pituitary program. Interassay variation was less than 15% and intra-assay coefficient of variation was lower than 10%. Results were expressed as the mean ± SEM. Statistical significance was assessed by Duncan's new multiple range test. The potencies of the peptides, based on 4-point assays, were calculated by the factorial analyses of Bliss and Marks with 95% confidence limits (21).

RESULTS AND DISCUSSION

A novel series of analogs of human GH-RH(1-28)Agm was synthesized by solid-phase methodology, following the method of Kent (18), to shorten the time of the synthesis. Every synthesis was finished within 4–5 days depending on the number of the necessary repetitions

of incomplete coupling steps. The purity of crude peptides corresponded to that of our earlier synthesized peptides.

After reduction of Met(0) with thioglycolic acid in peptides that contain Met^{27} the analogs were purified by preparative HPLC. The purity of isolated peptides was greater than 95% on the basis of absorbance data obtained on analytical HPLC. Amino acid analyses of the pure products showed the expected amino acid compositions (±5%).

The peptides were tested *in vitro* for their ability to release GH from dispersed rat pituitary cells, as compared to GH-RH(1-29)NH_2. Table 1 presents the structures and relative *in vitro* GH-releasing potencies of the 12 new GH-RH(1-28)Agm analogs, the parent compound GH-RH(1-29)NH_2 and two previously prepared control peptides (MZ-2-51, MZ-2-75).

We reported earlier that some analogs of GH-RH(1-29)NH_2 containing Agm^{29} are very active (6–9). In addition to Agm substitution, all these peptides contained Nle in position 27. In order to determine whether the high GH-releasing potencies of these analogs are due to replacement of Arg^{29} by Agm or whether additional substitutions such as Nle^{27},Ala^{15},D-Ala^2 and Dat^1 play a role in the increased activities, we synthesized a series of peptides: hGH-RH(1-28)Agm (MZ-3-175), [Nle^{27}]GH-RH(1-28)Agm (MZ-3-179), [Dat^1, Ala^{15}]GH-RH(1-28)Agm (MZ-3-191), [Dat^1,Ala^{15}, Nle^{27}]GH-RH(1-28)Agm (MZ-2-51), (9), [D-Ala^2, Ala^{15}]GH-RH(1-28)Agm (MZ-3-201) and [D-Ala^2, Ala^{15}, Nle^{27}]GH-RH(1-28)Agm (MZ-3-195). *In vitro* results showed that replacement of Arg^{29} by Agm in the GH-RH(1-29)NH_2 molecule (MZ-3-175) caused a slight increase in the relative potency, 1.2. Introduction of [Dat^1,Ala^{15}] and [D-Ala^2,Ala^{15}] into GH-RH(1-28)Agm (MZ-3-175) led to MZ-3-191 and MZ-3-201 possessing relative activities of 8, 2 and 7.1, respectively. Nle^{27} substitution in GH-RH(1-28)Agm (MZ-3-179) and MZ-3-191 (MZ-2-51) increased the potencies to 3.8 and 10.5, respectively. However, the same substitution in MZ-3-201 (MZ-3-195) resulted in a decrease of relative potencies to one third, that is, 2.4. Apparently, the effect of replacement of Met^{27} by Nle depends on the *N*-terminal structure; it is advantageous in the presence of Dat^1-Ala^2 but is unfavorable when paired with Tyr^1-D-Ala^2. The relative potencies of peptides containing Agm at the *C*-terminus increase in the following order: GH-RH(1-29)NH_2 < GH-RH(1-28)Agm < [Nle^{27}]GH-RH(1-28)Agm < [D-Ala^2, Ala^{15}]-GH-RH(1-28)Agm < [Dat^1,Ala^{15}]GH-RH(1-28)Agm < [Dat^1, Ala^{15}, Nle^{27}]GH-RH(1-28)Agm.

The synthesis of analogs containing *N*-$MeTyr^1$ was based on the fact that incorporation of this amino acid together with D-Ala^2 and Ala^{15} into GH-RH(1-29)NH_2 yielded a very active analog (5). Our analogs that contain *N*-$MeTyr^1$ were less active than peptides with des-NH_2-Tyr in position 1. [N-$MeTyr^1$, D-Ala^2, Ala^{15}, Nle^{27}]GH-RH(1-28)Agm (MZ-3-213) displayed 2.6 times higher GH release than GH-RH(1-29)NH_2, but comparing its activity with that of MZ-2-51, replacement of Dat^1 by N-$MeTyr^1$ (MZ-2-217) decreased the potency to one-fourth.

The effects of the iv administration of GH-RH(1-29)NH_2, used as a reference standard, and of the analogs MZ-3-149, MZ-3-191, and MZ-3-201 in male rats 5 min after the injection are shown in Table 2.MZ-3-149, MZ-3-191, and MZ-3-201 induced GH release 7.6, 4.9, and 3.3 times as great as GH-RH(1-29)NH_2,

TABLE 1

In vitro *GH-releasing activities of GH-RH(1-29)Agm analogs as compared to hGH-RH(1-29)NH_2*

Code No.	Amino acids in position								Relative potency
	1	2	12	15	21	27	28	29	
hGH-RH(1-29)NH_2	Tyr	Ala	Lys	Gly	Lys	Met	Ser	$ArgNH_2$	1.0
MZ-3-145	Dat	Ala	Lys	Ala	D-Lys	Nle	Asp	Agm	1
MZ-3-149	Dat	Ala	Lys	Ala	Lys	Nle	Asp	Agm	5.6
MZ-3-175	Tyr	Ala	Lys	Gly	Lys	Met	Ser	Agm	1.2
MZ-3-179	Tyr	Aa	Lys	Gly	Lys	Nle	Ser	Agm	3.8
MZ-3-191	Dat	Ala	Lys	Ala	Lys	Met	Ser	Agm	8.2
MZ-3-195	Tyr	D-Ala	Lys	Ala	Lys	Nle	Ser	Agm	2.4
MZ-3-199	Dat	D-Ala	Lys	Ala	Lys	Nle	Ser	Agm	0.6
MZ-3-201	Tyr	D-Ala	Lys	Ala	Lys	Met	Ser	Agm	7.1
MZ-3-213	*N*-MeTur	D-Ala	Lys	Ala	Lys	Nle	Ser	Agm	2.6
MZ-3-217	*N*-MeTyr	Ala	Lys	Ala	Lys	Nle	Ser	Agm	3.4
MZ-3-221	*N*-MeTyr	Ala	D-Lys	Ala	D-Lys	Nle	Ser	Agm	1.4
MZ-3-225	*N*-MeTyr	D-Ala	Lys	Ala	D-Lys	Nle	Ser	Agm	1.5
MZ-2-75[a]	Dat	Ala	Lys	Ala	D-Lys	Nle	Ser	Agm	4.0
MZ-2-51[a]	Dat	Ala	Lys	Ala	Lys	Nle	Ser	Agm	10.5

[a] (9).

TABLE 2

Intravenous activity of GH-RH(1-29)-NH_2 and its Agm analogs in tests on GH release in male rats

Peptide	Dose (μg/kg)	Plasma GH (ng/mL)		Mean ± SEM
		0 min	5 min	15 min
Saline		76.5 ± 13.2	80.7 ± 16.7	32.1 ± 2.7
GH-RH(1-29)NH_2	0.4	78 ± 6.5	221.6 ± 46.3*	63.7 ± 18.5
	1.0	59.0 ± 13.3	450.4 ± 53.6**	121.0 ± 14.9*
Potency			1.0	1.0
MZ-3-149	0.2	81.2 ± 17.0	531.1 ± 129**	196.1 ± 39.1*
	0.5	67.2 ± 14.8	1796.6 ± 245**	355.0 ± 69.4**
Potency			7.6	20.9
95% limits			(3.4–16.7)	(1.5–292.3)
MZ-3-191	0.2	53.5 ± 8.3	352.5 ± 65.6[b]	124.4 ± 36.7[a]
	0.5	63.7 ± 10.4	501.4 ± 84.9[b]	107.8 ± 24.9[a]
Potency			4.9	4.3
95% limits			(1.3–18.6)	(0.8–22.9)
MZ-3-201	0.2	79.5 ± 13.3	109.6 ± 37.7[a]	42.1 ± 8.7
	0.5	71.0 ± 10.9	690 ± 93.1[b]	133.1 ± 22.1[a]
Potency			3.3	1.7
95% limits			(1.9 ± 5.8)	(0.9 ± 3.3)

Potencies of the analogs were calculated by the factorial analysis of Bliss and Marks with 95% confidence limits and were based on the doses of 0.4 and 1.0 μg/kg b.w. of GH-RH(1-29)-NH_2 and 0.2 and 0.5 μg/kg b.w. of the analogs.
[a] $P<0.05$ and [b] $P<0.01$ vs. saline.

respectively. When these analogs were compared to the parent hormone at 15 min post-injection, they showed potencies 20.9, 4.3, and 1.7 times higher, respectively. The intravenous activity of analog MZ-3-149 was higher than that of MZ-2-51, which induced GH release 4.4 times as great GH-RH(1-29)NH_2 at 5 min and 5.57 times higher at 15 min (8). Thirty min after the injection, only the GH levels in animals injected with MZ-3-149 at the dose of 0.5 μg/kg were higher ($P<0.05$) than in controls. The response to 0.5 μg/kg of MZ-3-149 at 5 min post-injection was significantly greater than the GH level induced by the same dose of MZ-3-191 and MZ-3-201 ($P<0.01$) or by 1.0 μg/kg of GH-RH(1-29)NH_2 ($P<0.01$). Both doses of MZ-3-149 (0.2 and 0.5 μg/kg) produced a significantly greater GH release than the standard ($P<0.01$) 15 min after the injection.

The sc activity of these peptides is shown in Table 3. The relative potencies of MZ-3-149, MZ-3-191 and MZ-3-201 compared to GH-RH(1-29)NH_2 (accepted as 1.0) were 63.7, 55.2, and 56.8 at 15 min and 57.6, 60.6, and 42.6, respectively, at 30 min post-injection. In previous tests (8) the sc activity of MZ-2-51 was 34.3 times greater than that of GH-RH(1-29)NH_2 at 15 min. At 60 min, only MZ-3-149 showed significant activity ($P<0.05$) compared to controls at the dose of 0.5 μg/kg. These results indicate that the Agm analogs MZ-3-149, MZ-3-191, and MZ-3-201 are much more potent than the parent hormone when they are injected sc or iv. Furthermore, MZ-3-149 shows a more prolonged GH release than GH-RH(1-29)NH_2. These findings indicate that these analogs are resistant to local degradation at the site of injection and suggest that they are also less susceptible to enzymatic inactivation in the bloodstream and/or that they possess a greater affinity for the GH-RH receptors than GH-RH(1-29)NH_2.

Our work shows that in addition to Agm in position 29, substitutions in other positions, e.g. 1, or 2, 15, 27 and/or 28 can lead to analogs with increased GH-releasing activities.

Analogs of GH-RH(1-28)Agm containing Dat in position 1 or D-Ala in position 2, plus Ala^{15} display peptides with very high activity. Substitution of Met^{27} by Nle increased the potency further provided it was purified with Dat^1. Replacement of Ser^{28} by Asp caused a certain decrease of the activity *in vitro* (Table 2). Although among our new analogs [Dat^1,Ala^{15}]GH-RH(1-28)Agm (MZ-3-191), [D-Ala^2,Ala^{15}]GH-RH(1-28)Agm (MZ-3-201), and [Dat^1,Ala^{15},Nle^{27},Asp^{28}] GH-RH(1-28)Agm (Mz-3-149) showed, in that order, the highest activity *in vitro*, they were less active than the Nle^{27} homolog of MZ-3-191 (MZ-2-51) or our previous series (9).

These four peptides were also the most active *in vitro* but in an entirely different potency order. Previously, we reported that our agmatine analog [desNH$_2$-Tyr^1, Ala^{15}, Nle^{27}]GH-RH(1-28)Agm (MZ-2-51) was 4.4 and 34.4 times more potent than GH-RH(1-29)NH_2 when it was injected iv and sc, respectively (8). Our results indicate that the new agmatine analog MZ-3-

TABLE 3

Subcutaneous activity of GH-RH(1-29)-NH_2 and its Agm analogs in tests on GH release in male rats

Peptide	Dose (μg kg)	Plasma GH (ng/mL)		Mean ± SEM
		0 min	15 min	30 min
Saline		78.0 ± 12.3	6.4 ± 15.6	62.4 ± 12.4
GHRH(1-29)NH_2	50	69.4 ± 13.8	177.3 ± 22.0[b]	125.2 ± 10.0[a]
	150	60.0 ± 12.8	462.2 ± 25.9[b]	148.5 ± 48.3[a]
Potency			1.0	1.0
MZ-3-149	1.5	77.8 ± 11.3	273.6 ± 78.3[b]	106.1 ± 10.3[a]
	4.5	74.8 ± 10.7	794.4 ± 129[b]	207.0 ± 20.0[b]
Potency			63.7	57.6
95% limits			(40.0–100.3)	(24.1–137.7)
MZ-3-191	1.5	47.2 ± 8.9	255.3 ± 5.0**	119.3 ± 19.0*
	4.5	68.8 ± 9.8	736.2 ± 112**	223.5 ± 29.0*
Potency			55.2	60.6
95% limits			(36.1–84.5)	(17.9–205.2)
MZ-3-201	1.5	73.8 ± 11.3	290.3 ± 36.2**	117.5 ± 15.0*
	4.5	78.0 ± 9.8	672.2 ± 146**	173.8 ± 23.7**
Potency			56.8	42.6
95% limits			(31.2–103.3)	(7.7–235.6)

Potencies of the analogs were calculated by the factorial analysis of Bliss and Marks with 95% confidence limits and were based on the doses of 50 and 150μg/kg b.w. of GH-RH(1-29)-NH_2 and 1.5 and 4.5 μg kg b.w. of the analogs.
[a] $P<0.05$ and [b] $P<0.01$ vs. saline.

149, which is structurally similar to MZ-2-51, but contains Asp in position 28 in place of Ser (Table 1) is more potent than GH-RH(1-29)NH_2, and also more potent than MZ-3-191 and MZ-3-201. Thus, MZ-3-149 with Asp^{28} appears to be at present the most potent analog *in vivo* and more active than its Ser^{28} homolog, MZ-2-51, which is the most potent analog *in vitro*. While activity *in vitro* mainly depends on the binding capacity of the peptides to their receptors, high *in vivo* potency also requires the fulfilling of some additional criteria, such as favorable transport properties, suitable binding the plasma proteins, enhanced ability to permeate various barriers and metabolic stability. These latter criteria seem to be met by the zwitterionic $Asp(0^-)^{28}$-$Agm(H^+)$ portion of MZ-3-149 to an extent that recompenses for the higher binding capacity of MZ-2-51 rendered by its basic Ser^{28}-$Agm(H^+)$ moiety.

Although further investigations are necessary to determine the acute and chronic effects in other animal species and in humans, it is likely that our highly active GH-RH(1-28)Agm analogs, especially MZ-3-149, will find important clinical and veterinary applications.

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