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Potent Trypsin-resistant hGH-RH Analogues

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Abstract: A series of analogues of hGH-RH-(1–29)-NH_2 designed to have metabolic stability has been synthesized. Standard Boc-SPPS was employed, modified to permit the guanidinylation of amino side-chains after chain assembly but before release from the resin. [Dat^1, $Har^{11,12,20,21,29}$, Ala^{15}, Nle^{27}, Asp^{28}]-, [Dat^1, $Har^{11,20,29}$, Orn^{12}, Ala^{15}, Nle^{27}, Asp^{28}]-, and [Dat^1, $Gap^{11,12,21,29}$, Ala^{15}, Har^{20}, Nle^{27}, Asp^{28}]-hGH-RH-(1–29)-NH_2 were completely resistant to trypsin and about 50 times as potent as hGH-RH-(1–29)-NH_2 itself when injected subcutaneously in rats. These peptides are candidates for clinical application in the therapy of GH deficiency. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: hGH-RH analogues; rGH secretion; homoarginine; 3-guanidino-2-aminopropionic acid; 4-guanidino-2-aminobutyric acid; guanidinylation; solid-phase peptide synthesis

INTRODUCTION

The use of human growth hormone (hGH), produced by recombinant DNA methods, in the therapy of GH insufficiency, both in children and in elderly subjects is generally accepted, but an alternative form of therapy based on human growth hormone-releasing hormone (hGH-RH), that stimulates secretion of endogenous hGH, could also be used. It has been demonstrated that therapy with hGH-RH can be equivalent to that with hGH in terms of linear growth in children [1], and also can reverse age-related decreases in hGH and insulin-like growth factor in men over 60 years old [2], suggesting that prolonged treatment could improve the health, strength and functional capacity of the aging population. Further, application of hGH-RH does not produce a 'pharmacological' rise in hGH level giving a wider margin of safety than hGH administration.

The major problem of the therapy based on hGH-RH or hGH-RH-(1–29)-NH_2 is their susceptibility to enzyme cleavage. It has been demonstrated that degradation at the site of injection after subcutaneous administration is extremely rapid [3]. There is a great demand for more stable analogues which would allow a reduction in dose and frequency of administration. Numerous analogues of hGH-RH-(1–29)-NH_2 have been synthesized and tested in a search for peptides of increased metabolic stability and potency [4–6]. hGH-RH-(1–29)-NH_2 itself is rapidly metabolized in plasma, as a result of Ala^2-Asp^3 peptide bond cleavage by dipeptidylpeptidase IV with the formation of an inactive 3–29 fragment [7]. This cleavage is completely suppressed by introduction of a desaminotyrosine (Dat) residue at position 1 instead of tyrosine [8–10]. The other metabolites observed were characteristic of trypsin-like cleavages at Arg^{11}-Lys^{12} and Lys^{12}-Val^{13} [11]. It has been observed in this laboratory that [Nle^{27}] hGH-RH-(1–29)-NH_2 is cleaved by trypsin at all basic amino acid residues, including the *C*-terminal amide bond, but an analogue with Orn for Lys

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replacements was cleaved only at the Arg residues [11]. This result was consistent with the high biological activity *in vivo* of analogues containing Orn in positions 12 and 21 [10]. Recently several biologically active analogues containing side-chain extended homoarginine (Har) instead of Arg and Lys were described. It was found that peptide bonds formed by the carboxyl group of Har are completely stable to trypsin [6].

The aim of the present work was to examine the possibility of obtaining biologically active analogues, similar to those previously described [6], by replacing all the Arg and Lys residues with non-coded amino acid residues containing a guanidino group in the side-chain: Har, 4-guanidino-2-aminobutyric acid (Gab) or 3-guanidino-2-aminopropionic acid (Gap). Orn and D-Arg residues were also incorporated. The structures of the synthesized analogues are shown in Table 1.

MATERIALS AND METHODS

hGH-RH-(1–29)-NH_2 was purchased from Sigma Chemical Co. Boc-Dap-(Fmoc)-OH and Boc-Dab-(Fmoc)-OH were purchased from Senn Chemicals.

Peptide Chain Assembly

Amino acids except arginine were of the L-configuration. α-Amino groups were Boc protected, and side-chains were as follows: Asp, cyclohexyl; Lys, 2,4-diaminobutyric acid and 2,3-diaminopropionic acid, Fmoc; Orn, Z; Ser and Thr, Bzl; and Tyr, Z(2-Br). The hydroxy group of Dat was not protected. MBHA resin (0.818 g, 0.4 meq; Bachem California, 0.52 meq/g) was used after being swelled in DCM for 30 min, treated with 5% DIEA (1 × 1 min, 1 × 20 min) and washed with DCM (6 × 1 min). The synthesis of the protected peptide-resin was carried out according to the standard procedures consisting of the following: (a) deprotection with 55% TFA in DCM (1 × 1 min, 1 × 20 min); (b) washing with DCM (3 × 1 min); (c) washing with 30% dioxane in DCM (2 × 1 min); (d) washing with DCM (3 × 1 min) (e) neutralization with 5% DIEA in DCM (1 × 1 min, 1 × 5 min); (f) washing with DCM (6 × 1 min); (g) coupling of Boc-amino acid (1.2 mmol) in the presence of DIC (1.2 mmol) in DCM for 2 h, in the case of Boc-Gln and Boc-Asn HOBt (1.2 mmol) was added to the reaction mixture; (h) washing with DCM (6 × 1 min).

Table 1 Structures of Analogues Compared with hGH-RH-(1–29)-NH_2, (Tyr[1]-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg[11]-Lys[12]-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg[20]-Lys[21]-Leu-Leu-Gln-Asp-Ile-Met[27]-Ser-Arg[29]-NH_2)[a]

Peptide	Position of residue							M (formula)	ESI-MS found (calcd for $M + nH^+$)		
	1	11	12	20	21	27	29		$M + 3H^+$	$M + 4H^+$	$M + 5H^+$
hGH-RH-(1-29)-NH_2	Tyr	Arg	Lys	Arg	Lys	Met	Arg				
1	Dat	Har	Har	Har	Har	Nle	Har	3492.9 ($C_{157}H_{258}O_{43}N_{47}$)	1165.4 (1165.0)	874.0 (874.0)	699.4 (669.4)
2	Dat	Har	Orn	Har	Orn	Nle	Har	3380.9 ($C_{153}H_{251}O_{43}N_{47}$)	1127.9 (1128.0)	846.2 (846.2)	676.9 (677.2)
3	Dat	Gab	Gab	Gab	Gab	Nle	Gab	3351.8 ($C_{147}H_{238}O_{43}N_{47}$)	1118.6 (1118.3)	839.2 (839.0)	671.6 (671.4)
4	Dat	Har	Gab	Gab	Gab	Nle	Gab	3379.8 ($C_{149}H_{242}O_{43}N_{47}$)		846.3 (846.0)	677.2 677.0
5	Dat	Gab	Har	Har	Har	Nle	D-Arg	3451.0 ($C_{154}H_{253}O_{43}N_{47}$)	1152.0 (1151.3)	863.8 (863.8)	691.6 (691.2)
6	Dat	Har	Gab	Har	Har	Nle	D-Arg	3451.0 ($C_{154}H_{253}O_{43}N_{47}$)	1151.4 (1151.3)	863.6 (863.8)	691.3 (691.2)
7	Dat	Gap	Gap	Gap	Gap	Nle	Gap	3282.7 ($C_{142}H_{229}O_{43}N_{47}$)	1095.3 (1095.2)	821.7 (821.7)	657.8 (657.5)
8	Dat	Gap	Gap	Har	Gap	Nle	Gap	3324.8 ($C_{145}H_{235}O_{43}N_{47}$)	1109.6 (1109.3)	832.2 (832.2)	666.0 (666.0)

[a] In all analogues Ala was in position 15, and Asp in position 28.

Introduction of guanidino group. Protected peptide-resin was treated with 50% piperidine in DMF (1 × 10 min, 1 × 2 h), then washed with DMF (3 × 1 min), 50% DMF in DCM (2 × 1 min), 50% methanol in DCM and DCM (3 × 2 min). The resulting protected peptide-resin was treated with *N*,*N*′-bis-Boc-*S*-methylisothiourea (five molar excess) and 4-dimethylaminopyridine (70 mg) in DMF for 4 days. The product was washed with DMF (3 × 1 min), DCM (3 × 1 min) and treated with 55% TFA in DCM (1 × 1 min, 1 × 20 min, 1 × 40 min), and washed with DCM (3 × 1 min), 50% DMF in DCM (2 × 1 min) and DCM (2 × 1 min). The yield was 2.0–2.5 g.

Cleavage of the peptide from the resin. A sample (500 mg) of protected peptide-resin was treated with liquid HF (10 ml) in the presence of anisole (1 ml) for 1 h at 0 °C. The HF was removed under reduced pressure and the residue was treated with cold ether, extracted with 50% acetic acid and lyophilized.

Purification. The crude peptides (20 mg samples) were purified using a Knauer HPLC system with a Vertex column Nucleosil-300 C_{18} (8 × 200 mm, 5 μm); solvent system: A; 0.1% TFA in water, B; 80% MeCN in A. Elution: 20%–55% B in 30 min, then 55% B in 30 min. The flow rate was 2 ml/min. Fractions were analysed on a Vertex column Nucleosil-100 C_{18} (4 × 250 mm, 5 μm) using a gradient of 25%–70% in 30 min; flow rate 1 ml/min; detection at 220 nm. Homogeneous fractions containing one peak were combined and lyophilized. Fractions were pooled for maximum purity rather than yield. The yield of homogeneous product was 1.0–4.3 mg. Structures were confirmed by ESI-MS spectra (a Finnigan MAT 95S spectrometer, Bremen, Germany) (Table 1).

Tryptic hydrolysis. A sample of peptide (1.2 mg) was dissolved in ammonium acetate buffer (2.9 ml, 0.05 M, pH 8.5) and incubated at 37 °C for 20 min. Then a solution of trypsin (100 μl, 0.02 mg/ml; Serva, 36 U/mg) was added. The resulting solution was incubated at 37 °C for 60 min. A sample (500 μl) of the solution was diluted with 0.5 M acetic acid (1 ml) and lyophilized. An analysis of the resulting material was performed by HPLC using a Knauer system with a Eurospher 100 C_{18} (4 × 250 mm, 5 μm) column; solvent system: 0.1% TFA in water, B: 80% MeCN in A; linear gradient 25%–70% B in 30 min; flow rate 1 ml/min; detection at 220 nm. Peptides **1, 2** and **8** were completely stable. In the case of peptides **3–7** slow digestion was observed but over 60% of the peptides remained unchanged. Under these conditions [Nle^{27}]-hGH-RH-(1–29)-NH_2 was completely digested [11].

Bioassays

Male Wistar rats (240–260 g) were anaesthetized with an intraperitoneal injection of ketamine (120 mg/kg) and the jugular vein was chronically cannulated for blood collection. Thirty minutes later the test substances in saline were injected subcutaneously: hGH-RH-(1–29)-NH_2 in doses of 50 and 150 μg/kg of body weight, and the analogues in doses of 1 and 3 μg/kg of body weight. A control group of rats was injected with saline only. Blood samples (approximately 0.2 ml) were drawn at 0, 15 and 30 min. The blood samples were centrifuged, and the plasma samples were separated and stored at −20 °C until assayed by RIA methods. The plasma GH concentration was measured with an RIA method using kits provided by Biotrak (Amersham Life Science, England).The concentration of GH was determined in 0.05 ml aliquots of the plasma samples and only the samples containing a high level of GH in aliquots of 0.0025 ml were reanalysed. The sensitivity for rat GH was 0.16 ng/tube. The intra- and inter-assay coefficients of variation were 6% and 11%, respectively. The intra-assay coefficients of variation for duplicate of control samples were lower than 10%.The results for two experiments (firstly for analogue **1** and secondly for **2** and **8**) are shown in Table 3, and illustrated in Figures 1 and 2, respectively. The effects of hGH-RH-(1–29)-NH_2, analogue **2** and analogue **8** on plasma concentrations of corticosterone were determined by RIA with a commercial kit (ICN Biomedicals, Costa Mesa, CA). The concentration in all plasma samples was determined using 0.01 ml aliquots. The sensitivity of this assay was 25 ng/ml. The intra-assay coefficients of variation for duplicates of control samples were lower than 10%. Injection of these peptides had no effect on corticosterone level.

RESULTS AND DISCUSSION

Eight novel analogues of hGH-RH-(1–29)-NH_2 containing homoarginine (Har), Orn, Gab, Gap and D-arginine residues instead of Arg and Lys were synthesized by solid-phase methodology. These replacements were designed to protect the molecules from digestion by trypsin-like proteases. The positions occupied by Arg and Lys residues are known

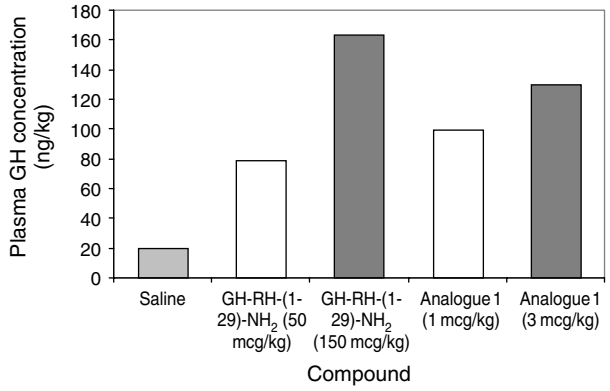
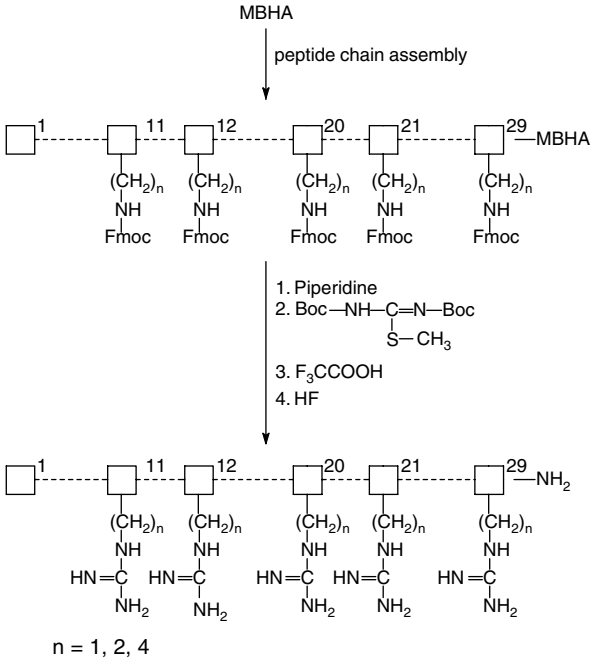


Figure 1 Plasma GH concentration 15 min after the injection of saline, hGH-RH-(1-29)-NH_2 and analogue **1**.



to be processing sites of cleavage by trypsin-like proteases in the native peptide. An Orn residue had been introduced at positions 12 and 21 of hGH-RH-(1–29)-NH_2 previously [10] resulting in an increase of biological activity, and recently it was demonstrated that introduction of Orn decreased the number of degradation products after treatment with trypsin [11]. It was our hope that the dibasic residues selected for these studies would give trypsin-resistant analogues. A distinctive feature of their synthesis (Scheme 1) was the use of commercially available Boc derivatives of dibasic amino acids in which the side chain amino functions were Fmoc protected. This latter protection was removed after peptide chain assembly by the treatment with piperidine and then the peptide-resin conjugate was guanidinylated. Guanidinylation was performed using *N*,*N*′-bis(Boc)-*S*-methylisothiourea

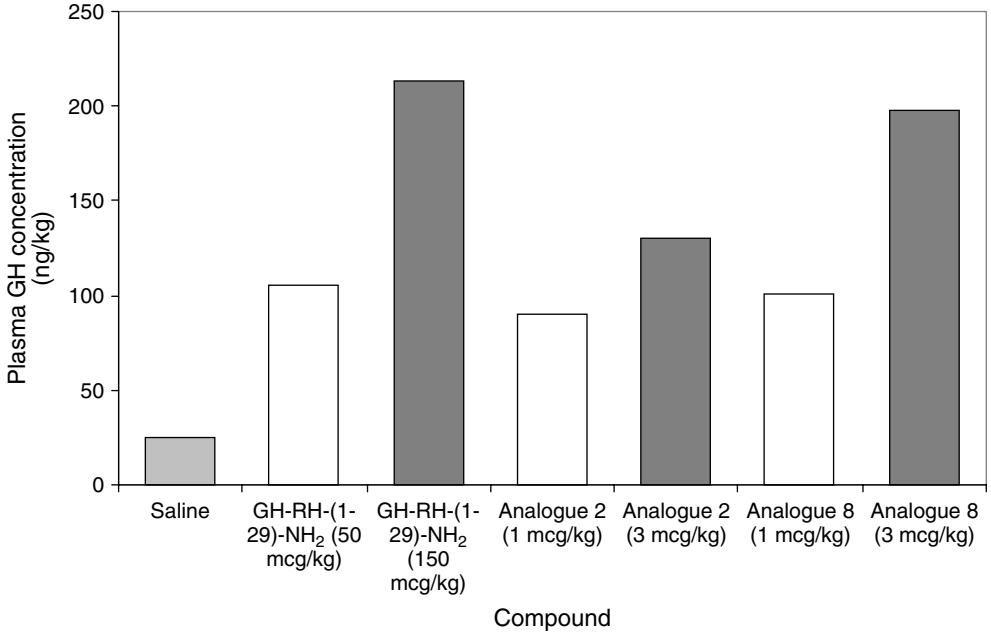


Figure 2 Plasma GH concentration 15 min after the injection of saline, hGH-RH-(1-29)-NH_2 and analogue **2** and analogue **8**.

with a catalytical amount of 4-dimethylaminopyridine. This process was monitored using the Kaiser colour test, and judged to be finished when free amino groups were no longer detected. The peptide was deprotected and cleaved from the resin by treatment with liquid HF. The crude product was purified to homogeneity by RP-HPLC. The peptides were subjected to digestion by trypsin under the conditions in which analogues containing Arg and Lys had been completely digested [11].

The results presented in Table 2 indicate that all these substitutions dramatically increase the resistance of these peptides to tryptic digestion. Under the conditions of complete digestion of the standard peptide the peptides remained untouched (**1, 2** and **8**) or only a portion was hydrolysed after 30 min digestion (**3, 4, 5, 6** and **7**). Since GH is released in a pulsatile manner and a higher level of GH is observed between 15 and 30 min after subcutaneous administration of GH-RH analogues, hydrolysis by trypsin-like enzymes could not affect the result of stimulation. Since these peptides contain Dat at position 1, it could be concluded that they are also resistant to dipeptidylpeptidase IV, which is responsible for degradation at the *N*-terminus.

Table 2 Survival of hGH-RH Analogues after Treatment with Trypsin

Analogue	Digestion time (min)	% of analogue remaining
1	30	>99
	60	>99
2	30	>99
	60	>99
3	30	86
	60	72
4	30	82
	60	67
5	30	80
	60	51
6	30	85
	60	73
7	30	75
	60	68
8	30	>99
	60	>99
[Nle^{27}]hGH-RH-(1-29)-NH_2	30	0
	60	0

Peptides **1, 2** and **8** were tested for their ability to stimulate GH release in rats by injecting them subcutaneously. The potency was compared with that of hGH-RH-(1–29)-NH_2. Doses of 1 and 3 μg

Table 3 Effect of Subcutaneous Administration of hGH-RH-(1–29)-NH_2 and its Analogues on GH Release in Male Rats

Compound	Dose (μg/kg)	Number of rats	Plasma GH concentration after injection (ng/ml)		
			0 min	15 min	30 min
Experiment 1 (see Figure 1)					
Saline		11	21.4 ± 2.9	19.8 ± 3.9	17.8 ± 2.7
hGH-RH-(1-29)-NH_2	50	11	24.5 ± 2.7	79.1 ± 7.9[a]	39.0 ± 5.9[a]
	150	11	27.1 ± 3.2	163 ± 18[b]	103 ± 15[b]
Analogue **1**	1	9	23.1 ± 2.0	99.1 ± 16[b]	42.5 ± 9.2[a]
	3	9	28.3 ± 4.1	130 ± 16[b]	58.4 ± 8.9[b]
Experiment 2 (see Figure 2)					
Saline		11	23.5 ± 1.5	25.3 ± 1.5	23.8 ± 1.8
hGH-RH-(1-29)-NH_2	50	9	27.1 ± 1.5	105.3 ± 6.7[b]	83.5 ± 4.7[b]
	150	9	29.3 ± 1.5	213 ± 17.7[b]	136.7 ± 12.2[b]
Analogue **2**	1	11	25.5 ± 1.1	89.8 ± 4.1[b]	89.8 ± 6.2[b]
	3	10	28.1 ± 1.9	130.6 ± 7.1[b]	110.3 ± 9.5[b]
Analogue **8**	1	10	25.4 ± 1.4	101.2 ± 5.7[b]	89.0 ± 4.0[b]
	3	10	27.4 ± 1.8	198.3 ± 11.1[b]	138.3 ± 9.5[b]

The results are expressed as mean ± SEM.
[a] $p < 0.01$ vs saline control.
[b] $p < 0.001$ vs saline control.

of the analogues per kg of body weight, and doses of 50 and 150 μg of hGH-RH-(1–29)-NH_2 per kg of body weight were injected. The results are shown in Table 3 and illustrated in Figures 1 and 2. It can be seen that the peptides studied stimulated the release of GH at 50 times lower doses than that of hGH-RH-(1–29)-NH_2. Since it has been demonstrated [1] that doses of 20 μg/kg/injection of hGH-RH stimulated linear growth in children and appeared equivalent to GH therapy, it is reasonable to conclude that doses of about 1 μg/kg/injection could be used in initial studies to examine the application of these peptides in the therapy of GH-deficient children.

It also should be noticed that synthesis of these peptides was performed with the use of a standard, commercially available resin. Previously reported superactive hGH-RH analogues contained agmatine at the *C*-terminus and for their synthesis, the preparation of an agmatine-resin was needed [10].

It is noteworthy that GH-RH, in addition to binding to specific receptors, exerts a direct intracellular effect on the translocation of messenger RNA in somatotrophs [12]. In this connection, the development of stable GH-RH analogues has been postulated [13] to obtain probes to study internalization processes at cellular level. The peptides obtained in this work seem to be good candidates for this purpose.

CONCLUSIONS

It appears from this work that highly active analogues with increased enzymatic stability can be obtained by substitution of Lys and Arg residues by amino acid residues containing a guanidino group in a side-chain of varied length.

The high activity and stability strongly suggest that these analogues could be candidates for further biological studies and also for clinical use to treat GH deficiency by promoting the secretion of endogenous GH.

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