

A method for evaluation of activity of growth hormone-releasing hormone analogues

Jan IZDEBSKI¹, Danuta KUNCE¹, Ewa WITKOWSKA¹, Ewa WOLIŃSKA-WITORT²,
Magdalena CHMIEŁOWSKA² & Bogusława BARANOWSKA²

1. Peptide Laboratory, Department of Chemistry, Warsaw University, Warsaw, Poland.
2. Neuroendocrinology Department, Medical Centre of Postgraduate Education, Warsaw, Poland.

Correspondence to: Bogusława Baranowska MD., PhD.
Neuroendocrinology Dept.
Marymoncka 99/103, 01-813 Warsaw, Poland
PHONE: +48 22 569 38 50
FAX: +48 22 569 38 59
EMAIL: zncmkp@op.pl; zne@cmkp.edu.pl

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Abstract

OBJECTIVE: It has been found that hGH-RH analogues with increased resistance to enzymatic degradation have a much higher potency than the native hGH-RH (1-29)-NH₂ and have an ability to partially reverse growth hormone deficiencies.

THE AIM: The aim of these studies was to elaborate a method which can be used for preliminary evaluation of new GH-RH analogues both from the point of view of their potency to release GH and enzymatic stability.

METHOD: Two highly active GH-RH analogues with increased resistance to trypsin-like enzymes, and hGH-RH(1-29)-NH₂ used as a standard, in doses 1 nM, 10 nM, and 100 nM were added to pituitary rat cell culture, and medium was collected after 30, 60, 120 and 240 min. GH concentration was measured by RIA kit.

RESULTS: It was observed that the potency of these two GH-RH analogues was several times higher than that of native compound. Moreover, the stimulation was much longer. This suggests that high activity of these analogues *in vivo* could be the result of increased enzymatic stability.

CONCLUSION: This method can be used for selecting more potent and more stable releasing peptides before *in vivo* evaluation.

INTRODUCTION

The wide availability of human growth hormone (GH) produced by recombinant DNA methods has made possible the treatment of GH-deficient patients. However, in part of the cases GH insufficiency is due to changes in the control of GH secretion by the hypothalamus, rather than to decrease in somatotroph response caused by an impairment at the pituitary level [1] thus, an alternative form of

therapy based on the human hypothalamic growth hormone releasing hormone (hGH-RH) that stimulates GH secretion could also be used. This was supported by the observation that there was no consistent evidence of desensitization or depletion effect of therapy on the GH responses to GH-RH [1–3]. It has been demonstrated that native hGH-RH and the shortened fragment, hGH-RH(1-29)-NH₂, accelerate growth velocity in GH-deficient children [2]. Although the therapeutic usefulness of hGH-

RH, its shorter fragment and several analogues [4] has been demonstrated, susceptibilities to enzyme cleavage limit their use as effective drugs. The promising method to increase stability of these peptides is to introduce modifications which eliminate enzymatic cleavage sites. One solution is the introduction of unnatural amino acids instead of lysine and arginine which are processing sites of trypsin-like enzyme [5,6]. With the use of one of these potent peptides, JI-38, partial reversibility of growth hormone deficiency was reversed by long-term treatment [7].

This finding prompted us to elaborate a simple procedure which allows to evaluate new peptides from the point of view of their activity and enzymatic stability. Although the final evaluation always has to be made *in vivo* prior to clinical tests, for screening purposes *in vitro* bioassays are simpler, faster and less expensive. The classical bioassay using surviving rat pituitary is suitable for preliminary evaluation of potency of GH-RH analogues, but this method is not suitable for testing the dynamics of hormone release. Moreover, during several hours of incubation the test peptide, the released hormone and metabolic products accumulate in the culture medium and influence the metabolism of cell. Also, proteolytic enzymes may digest both the releasing peptide and the secreted hormone. We describe the procedure which avoids some of the shortcomings of classical method with the use of pituitary cell culture.

MATERIAL AND METHODS

In the study we used adult Wistar-Kyoto male rats (3-months old). The animals were maintained under standard laboratory conditions on 12/12 h light/dark cycle (lights on at 07.00 h). Food and water were available *ad libitum*. All experiments were approved by the First Warsaw Ethic Committee for Experiments on Animals.

The procedure of pituitary tissue dissociation, cell preparation and cell culture were based on methods described previously [8–10]. Briefly, pituitary glands were obtained from three-month-old (weight app. 260–280 g) male WKY rats, anesthetized by ketamine injection and decapitated. They were washed twice with DMEM, 2 mmol glutamine/l, penicillin (50 U/ml) and streptomycin (50 µg/ml) and processed for culture immediately. They were enzymatically dispersed during 20 min incubation at 37 °C in 0.1% trypsin followed by 20 min of incubation in 0.1% DNA-se I (deoxyribonuclease I from bovine pancreas, type IV) in DMEM with penicillin (50 U/ml) and streptomycin (50 µg/ml). The glands were finally mechanically dispersed on a sieve (50 mesh) and washed twice by centrifugation for 10 min at 50 × g with culture medium (DMEM containing 10% fetal calf serum). The pituitary cells were counted in a hemocytometer and assessed for viability by exclusion of trypan blue (>85%).

The pituitary cells (1×10^6 /ml) were incubated in 24-well culture plates for up to 48 hr in a humidified

atmosphere of 95% air and 5% CO₂ at 37 °C. The culture plates were washed with twice the volume of the serum-free medium 30 min before every experiment. The neuropeptides were dissolved in saline at concentrations 1 mM/l. They were diluted with serum-free medium to final nanomolar concentrations.

For short-term effects GH-RH analogues 1 and 2 as well as GH-RH(1-29)-NH₂ in doses 1 nM, 10 nM, 100 nM were added and then incubated for 30, 60, 120 and 240 min. The collected medium was stored at –20 °C until assayed for GH.

All media and chemicals were purchased from Sigma (Sigma Aldrich Chemie GmbH, Deisenhofen, Germany) and culture dishes from Corning (Costare, Corning Incorporated Corning, NY, USA).

Plasma rGH concentrations were measured with RIA methods using kits provided by Linco (Linco Research, USA).

The limit of detection was 1 ng/ml for GH. Intra-assay coefficients of variation were less than 9%.

GH-RH(1-29)-NH₂ analogues: Dat-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-*Har-Har*-Val-Leu-Ala-Gln-Leu-Ser-Ala-*Har-Har*-Leu-Leu-Gln-Asp-Ile-Nle-Asp-*Har*-NH₂ (analogue 1) and Dat-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-*Har-Orn*-Val-Leu-Ala-Gln-Leu-Ser-Ala-*Har-Orn*-Leu-Leu-Gln-Asp-Ile-Nle-Asp-*Har*-NH₂ (analogue 2), where Dat is desaminotyrosine, Har is homoarginine and Orn is ornithine, were prepared as described earlier [6]. GH-RH(1-29)-NH₂ itself was purchased from Sigma (Sigma Aldrich Chemie GmbH, Deisenhofen, Germany).

RESULTS

The effects of hGH-RH(1-29)-NH₂ analogues (1 and 2) on GH release from pituitary cells, compared with hGH-RH(1-29)-NH₂, are shown in Figures 1–4. Time course of GH concentration during incubation of peptides (100 nM) with rat pituitary cells is presented in Figure 5. It could be seen that after 30 min of incubation hGH-RH(1-29)-NH₂ (Figure 1 and Figure 5) stimulated release of GH more effectively than analogue 1 and 2. The results obtained after 60 min of incubation clearly indicate that concentration of GH in the culture stimulated with hGH-RH(1-29)-NH₂ is slightly lower and this drop in concentration is continued later, while in experiments with both analogue 1 and 2 GH content is substantially higher (Figure 2 and 5). The measurement performed up to 240 min indicate that GH content decreases in all experiments, but stronger stimulation of analogue 1 and 2 than that of hGH-RH(1-29)-NH₂ is observed (Figure 5).

DISCUSSION

For these studies of potency of hGH-RH(1-29)-NH₂ analogues to highly active peptides 1 and 2 were selected. The potency of these two peptides to promote release of

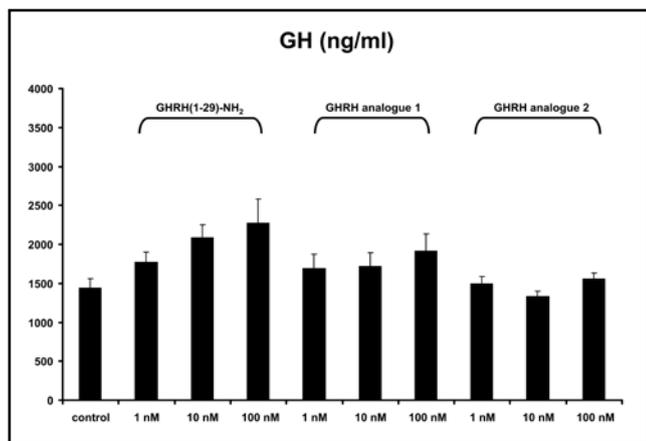


Figure 1. Effects of GH-RH analogues and GH-RH(1-29)-NH₂ on GH release after 30 mins.

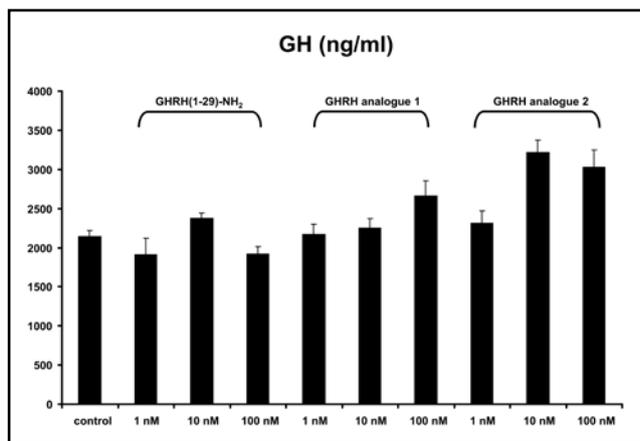


Figure 2. Effects of GH-RH analogues and GH-RH(1-29)-NH₂ on GH release after 60 mins.

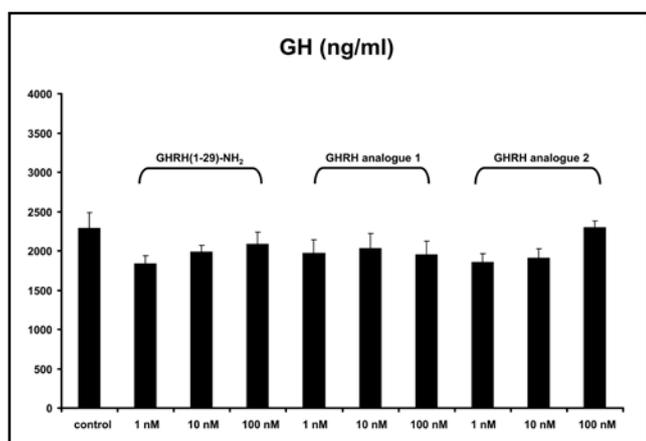


Figure 3. Effects of GH-RH analogues and GH-RH(1-29)-NH₂ on GH release after 120 mins.

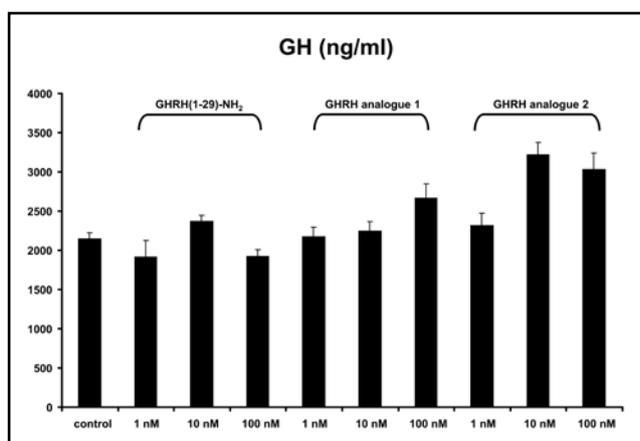


Figure 4. Effects of GH-RH analogues and GH-RH(1-29)-NH₂ on GH release after 240 mins.

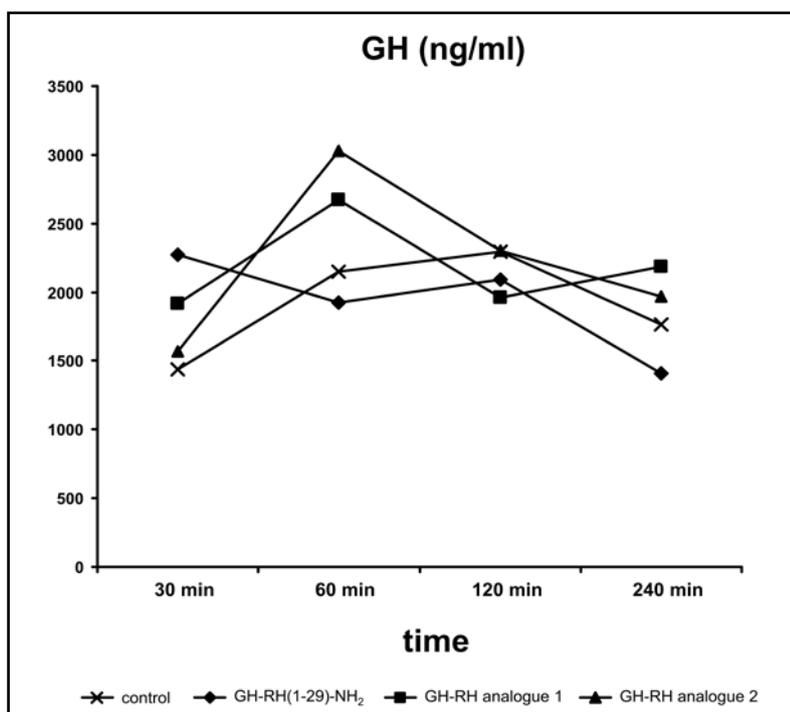


Figure 5. Effects of GH-RH analogues and GH-RH(1-29)-NH₂ (100 nM) on GH release after 30, 60, 120, 240 mins.

GH in pituitary cell culture was compared with that of hGH-RH(1-29)-NH₂ as a standard. The profiles of GH release involving these two peptides were different from that of the standard compound. Peptide 1 and 2 were more potent than the standard. Moreover, duration of action of these peptides was longer and was observed after 240 min, where activity of hGH-RH(1-29)-NH₂ was not observed. These results are consistent with our earlier studies in which we demonstrated that these analogues are about 50 times as potent as hGH-RH(1-29)-NH₂ itself when injected subcutaneously in rats, and are completely resistant to trypsin [6]. It seems that the decrease in concentration of GH during prolonged incubation could be mainly attributed to enzymatic degradation of the releasing peptide. It can be concluded that the described procedure may be useful for screening *in vitro* new compounds before more laborious and costly *in vivo* evaluation would be used.

CONCLUSIONS

The procedure for evaluation of new GH-releasing peptides is proposed which allows to access in one *in vitro* assay both potency and enzymatic stability.

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