Design, Synthesis, and Evaluation of a Long-Acting, Potent Analogue of Gonadotropin-Releasing Hormone

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The design, synthesis, and biological evaluation of a gonadotropin-releasing hormone (GnRH) agonist, [D-Lys^6(1,3,8-trihydroxy-6-carboxyanthraquinone)]GnRH ([D-Lys^6(Emo)]GnRH), is described. Synthesis of this analogue was carried out in a homogeneous solution as well as on a polymer support. [D-Lys^6(Emo)]GnRH was found to bind to rat pituitary GnRH receptors ($IC_{50} = 0.25$ nM), to induce luteinizing hormone (LH) release ($ED_{50} = 27$ pM), and to be devoid of any toxicity. This analogue also proved to be a very potent agonist in vivo and exhibited a prolonged bioactivity. Six hours after its administration to rats, LH levels were substantially higher than those of rats treated with a 10-fold higher dose of the parent peptide. Moreover, chronic treatment of adult male rats with [D-Lys^6(Emo)]GnRH (0.1 nmol/rat) for one week resulted in a further decrease of the weight of the testes and prostate as compared to those of rats that were treated with a higher dose of [D-Lys^6]GnRH (1 nmol/rat). The prolonged activity of [D-Lys^6(Emo)]GnRH may be attributed to its emodic acid moiety, which enhances the binding affinity of the analogue to human serum albumin. Indeed, we found that emodic acid binds to human serum albumin almost completely at the examined range of concentrations.

Introduction

Gonadotropin-releasing hormone (GnRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is a key integrator between the neural and the endocrine systems and plays a pivotal role in the regulation of the reproductive system. This neurohormone is synthesized in hypothalamic neurosecretory cells and is released in a pulsatile pattern into the hypothalamo−hypophyseal portal circulation. This pattern of GnRH secretion provokes the release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary, which, in turn, stimulate gonadal steroidogenesis and gametogenesis.[1,2] Chronic administration of GnRH or its superactive agonists results in down-regulation of GnRH receptors and desensitization of the pituitary gonadotrophs and thus causes the suppression of gonadotropin secretion.[3,4] Synthetic GnRH analogues, agonists as well as antagonists, have attracted remarkable interest because of their potential applications for the treatment of reproductive diseases, such as prostate and breast cancer, and their possible use as contraceptives.[5,6]

The mechanism of action of GnRH analogues in these diseases is believed to be at least partly related to gonadal steroid deprivation, which results from down-regulation and desensitization of the pituitary gonadotrophs. In cancer therapy, however, GnRH analogues have been demonstrated to exert direct inhibitory effects on the growth of cancer cells through GnRH receptors that are present in prostate, breast, and ovarian cancer.[5,7]

The relatively short half-life of GnRH in the general circulation (2−4 min)[5] is advantageous for the establishment of a pulsatile secretion pattern. However, potent agonists or antagonists having a prolonged bioactivity are certainly needed in the clinic for the induction of desensitization or contraception. Since the discovery of GnRH, more than 3000 analogues of the peptide have been synthesized and evaluated for their bioactivity. Most of the superagonists usually incorporate a D-amino acid substituting for Gly in position 6 and an *N*-ethylamide instead of the terminal Gly-NH_2 in position 10. These chemical modifications enhance the bioactive β-turn conformation of GnRH at the Gly^6−Leu^7 bond and decrease the susceptibility of the peptide to proteolytic degradation.[8,9] In addition, increasing the hydrophobicity of the peptide, by incorporation of appropriate amino acid residues, usually results in increased biological potency, probably due to a decrease in the rate of clearance from the general circulation and an increase in its apparent binding constant for binding to GnRH receptors.[10,11]

Conjugation of bulky moieties, such as tetramethylrhodamine, to the ϵ-amino group of [D-Lys^6]GnRH does not significantly affect the bioactivity of GnRH analogues.[12] Thus, in an attempt to produce effective targeted chemotherapy against cancer, several chemotherapeutic agents have been covalently attached to the ϵ-amino group of [D-Lys^6]GnRH.[13−15] For example, attachment of the cytotoxic compound 2-(hydroxymethyl)-anthraquinone hemiglutarate[16] (Figure 1) to [D-Lys^6]-GnRH has generated a powerful agonist, T-98, which bound to GnRH receptors on human breast cancer cells with a binding affinity similar to that of [D-Lys^6]GnRH. This analogue, however, exhibited a 5-fold higher biological activity than the parent peptide and inhibited the growth of human breast and prostate cancer.[14]

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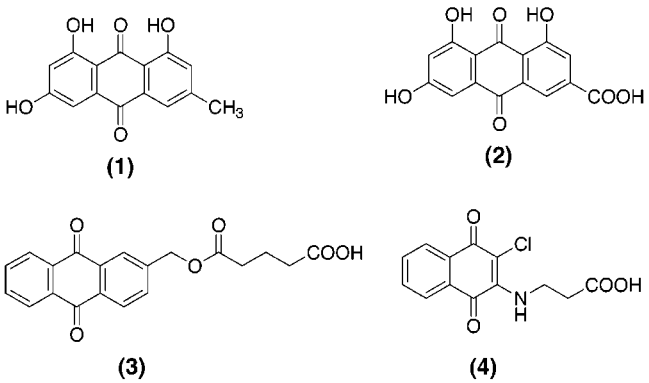

Figure 1. Chemical structures of the quinonic moieties: (**1**) emodin, (**2**) Emo, (**3**) AntrQ, (**4**) NQ.

In the present study we demonstrate the design and synthesis of a new long-acting, superactive GnRH conjugate that unlike T-98 does not exhibit any toxic or antiproliferative effects. For this purpose we have conjugated an emodin moiety (1,3,8-trihydroxy-6-methyl-9,10-anthraquinone) to [D-Lys^6]GnRH. Emodin is a naturally occurring polyhydroxylated anthraquinone that is widely used for preparation of laxatives. Furthermore, emodin has been demonstrated to bind with high affinity to serum albumin.[17] It has already been reported that binding of peptides to serum proteins may prolong their biological activity.[10,18]

Results

Chemistry. The synthesis of [D-Lys^6]GnRH conjugates that are modified at the ϵ-amino group of [D-Lys^6]-GnRH was carried out in two different routes. One way involved the reaction of the free ϵ-amino group of solid-phase-synthesized [D-Lys^6]GnRH, in a homogeneous solution, with the carboxylic functional group of the respective quinone moieties. This method employed (benzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate (PyBOP) as a coupling reagent and 4-methylmorpholine (NMM) as a base. Such a method is a "one-pot synthesis", which results in better yields and purity than other methodologies that use active esters, such as *N*-hydroxysuccinimide, as coupling reagents.[19] In the second route, [D-Lys^6]GnRH derivatives were prepared by employing an automatic multiple-peptide synthesizer, using Rink amide resin as the polymeric support and standard 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and corresponding reagents (Figure 2). Nevertheless, the routinely used Fmoc-D-Lys(Boc)-OH was replaced by Fmoc-D-Lys(Mtt)-OH, and the protected peptide was not cleaved from the resin. The Mtt group was removed selectively from the N^{ϵ}-amino group of D-Lys^6 by mild acidolysis (2% TFA in CH_2Cl_2) without affecting the other protecting groups.[20] Consequently, direct incorporation of emodic acid (1,6,8-trihydroxy-3-carboxyanthraquinone, Emo), 2-(hydroxymethyl)anthraquinone hemiglutarate (AntrQ), and *N*-(2-chloro-1,4-naphthoquinonyl)-β-alanine (NQ) (Figure 1) at the free N^{ϵ}-amino function of D-Lys^6 occurs by employing the standard coupling reagent and procedure. This reaction leads to the synthesis of [D-Lys^6-(1,3,8-trihydroxy-6-carboxyanthraquinone)]GnRH ([D-Lys^6(Emo)]GnRH), [D-Lys^6(2-(hydroxymethyl)anthraquinone hemiglutarate)]GnRH ([D-Lys^6(AntrQ)]GnRH), and [D-Lys^6(*N*-(2-chloro-1,4-naphthoquinonyl)-β-alanyl)]-GnRH ([D-Lys^6(NQ)]GnRH). We have synthesized the naphthoquinone and anthraquinone derivatives to evaluate the generality of the synthetic manipulations and as a reference for the biological activity of other conjugates of [D-Lys^6]GnRH. The corresponding molecular mass and the relative hydrophobicity of each of the conjugates are summarized in Table 1.

Table 1. GnRH Analogues: Molecular Mass, Relative Hydrophobicity, and Affinity of Binding (IC_{50}) to Rat Pituitary Receptors

peptide analogue	hydrophobicity (% B)[a]	MH^+ obsd (calcd)[b]	GnRH receptor binding IC_{50} ($\times 10^{-10}$ M)[c]
[D-Lys^6]GnRH	37	1254.6 (1254.4)	0.8 ± 0.01
[D-Lys^6(NQ)]GnRH	59	1516 (1516.07)	0.9 ± 0.01
[D-Lys^6(AntrQ)]GnRH	84	1589.7 (1588.7)	0.2 ± 0.02
[D-Lys^6(Emo)]GnRH	66	1537.6 (1536.7)	2.5 ± 0.1

[a] The percent of buffer B in which the analogue was eluted from the RP-18 column. [b] Observed (obsd) and calculated (calcd) *m*/*z* values of MH^+ monoisotopes. In most cases, an additional peak, corresponding to MNa^+, was observed. The purities of the synthetic peptides were usually >98%, according to two different analytical HPLC solvent systems as detailed in the Experimental Section. [c] IC_{50} = concentration of unlabeled ligand that displaces 50% of the bound tracer. Each point is the mean ± SEM of triplicates of one experiment out of three. For more details and abbreviations see Figures 1 and 3.

Biology. The ability of the [D-Lys^6]GnRH conjugates to bind to rat pituitary GnRH receptors was evaluated in vitro by displacement assays, using ^{125}I[D-Lys^6]GnRH as the radioligand. Figure 3 shows that incorporation of the quinone moiety in position 6 of the GnRH analogue does not change the high affinity of binding of the parent peptide to the GnRH receptors. Moreover, the affinity of binding of [D-Lys^6(NQ)]GnRH and of [D-Lys^6(AntrQ)]GnRH to GnRH receptors increased by 2- and 5-fold, respectively, whereas the affinity of binding of [D-Lys^6(Emo)]GnRH to the GnRH receptors was reduced by about 3-fold compared to that of the parent peptide. The binding affinities (IC_{50}) of the GnRH conjugates are summarized in Table 1.

To correlate the binding affinity of the newly synthesized GnRH conjugates with their bioactivity, these compounds were compared to [D-Lys^6]GnRH for LH-releasing capacity, using primary rat pituitary cell cultures. As shown in Figure 4, all conjugates exhibited enhanced LH-releasing activity compared to the parent peptide, in vitro. Notably, [D-Lys^6(Emo)]GnRH demonstrated the highest bioactivity, despite its lowest affinity of binding to the GnRH receptors (Figure 3). This bioactivity was completely inhibited by the antagonist [D-pGlu^1, D-Phe^2, D-Trp^{3,6}]GnRH, which reduced the [D-Lys^6(Emo)]GnRH-induced LH secretion to basal levels (Figure 5).

The above-mentioned properties of [D-Lys^6(Emo)]-GnRH prompted us to further evaluate its in vivo activity. Following intraperitoneal administration to intact rats, this conjugate proved to be a very potent agonist which induced an increase in serum LH levels similar to that of [D-Lys^6]GnRH, although the dose was reduced to 10% of that of the parent peptide (0.04 vs 0.4 nmol) (Figure 6). Moreover, the duration of the stimulation was also longer; 6 h after [D-Lys^6(Emo)]-

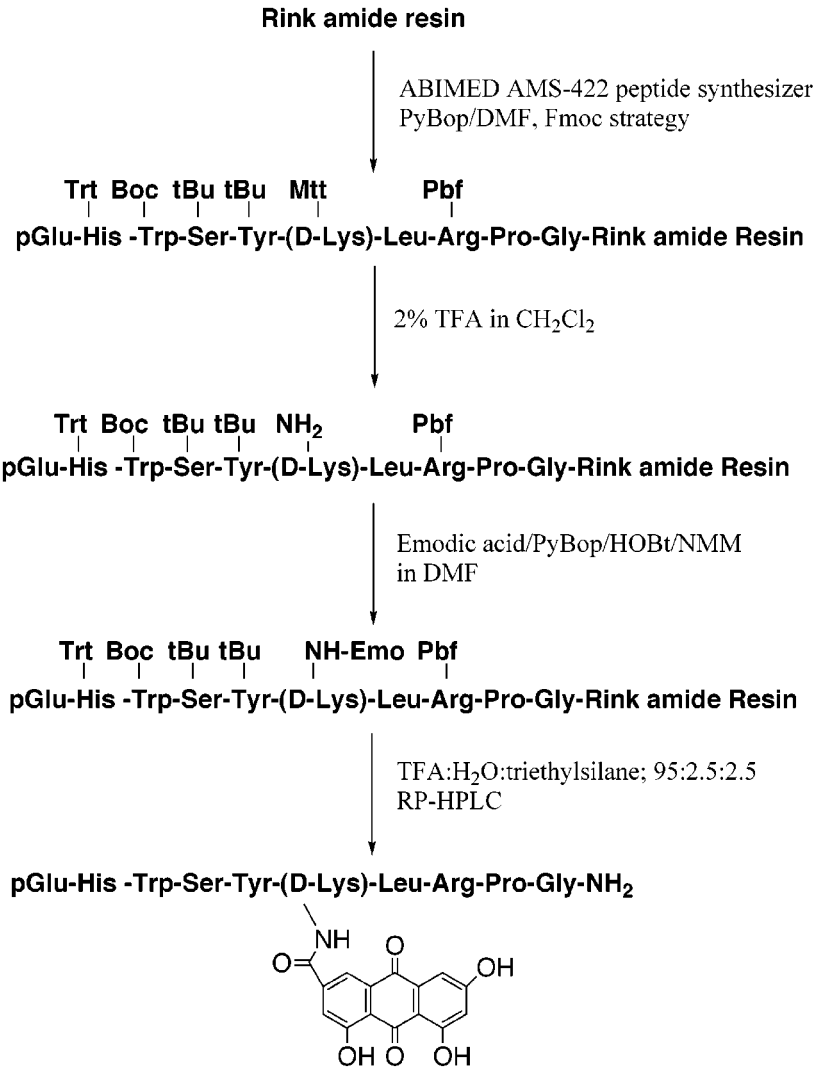

Figure 2. Solid-phase synthesis of [D-Lys^6(Emo)]GnRH.

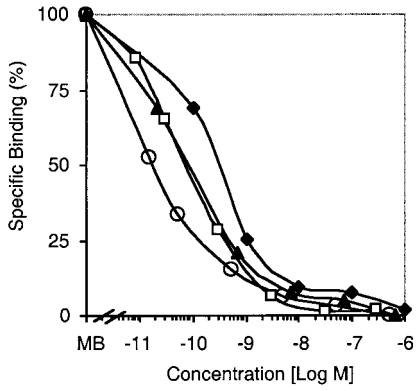

Figure 3. Displacement (%) of specific binding of ^{125}I[D-Lys^6]-GnRH from pituitary membranes of proestrous rats by increasing concentrations of unlabeled GnRH analogues [D-Lys^6-(AntrQ)]GnRH (○), [D-Lys^6(NQ)]GnRH (▲), [D-Lys^6(Emo)]GnRH (◆), and [D-Lys^6]GnRH (□). The membranes were incubated for 90 min at 4 °C with ^{125}I[D-Lys^6]GnRH and with the unlabeled peptides. Nonspecific binding was defined as binding of the labeled ligand in the presence of 1 μM [D-Lys^6]GnRH and was subtracted from the total binding for the calculation of specific maximal binding (MB). The results are the mean of two experiments carried out in triplicate. SEM values are omitted for clarity.

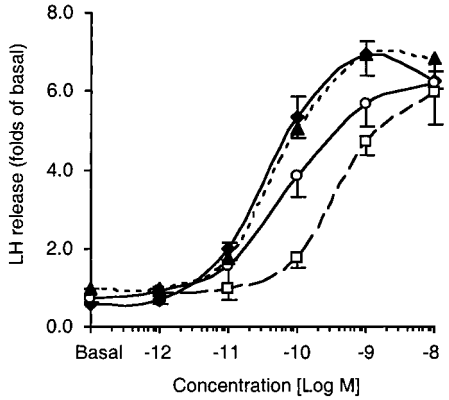

Figure 4. LH-releasing potency of [D-Lys^6]GnRH conjugates. Primary cultures of rat pituitary cells were incubated in M-199 containing the indicated concentrations of [D-Lys^6]GnRH (□), [D-Lys^6(AntrQ)]GnRH (○), [D-Lys^6(NQ)]GnRH (▲), or [D-Lys^6-(Emo)]GnRH (◆) for 4 h at 37 °C. The media were then collected, and the LH concentration was determined by RIA. The results are the mean ± SEM of LH concentrations of two experiments (four wells per experimental group). The basal release after 4 h was 9.41 ± 2 ng/mL.

GnRH administration, LH levels were about 6-fold higher than in the group of rats treated with [D-Lys^6]-GnRH. Administration of a higher dose of [D-Lys^6]GnRH (20 nmol/rat) induced a larger increase in serum LH levels. Furthermore, chronic administration of [D-Lys^6-(Emo)]GnRH (0.1 nmol/rat) to intact adult male rats for

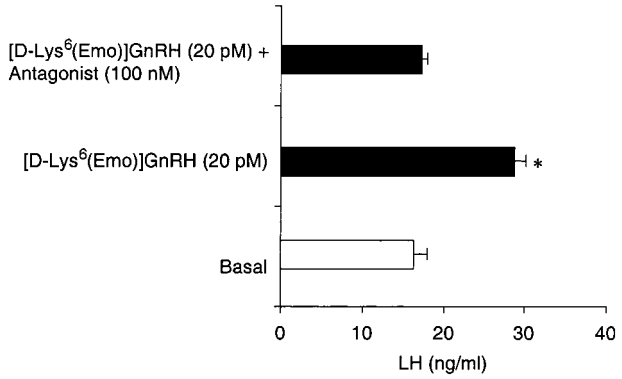

Figure 5. Effect of a GnRH antagonist ([D-pyr[1], D-Phe[2], D-Trp[3,6]]GnRH) on the induction of LH secretion from primary cultures of rat pituitary cells stimulated by [D-Lys[6](Emo)]-GnRH. The cells were incubated for 4 h at 37 °C with [D-Lys[6]-(Emo)]GnRH (20 pM) in the absence or presence of the GnRH antagonist (100 nM). The media were collected, and the LH concentration was determined by RIA. The results are the mean ± SEM of LH concentrations obtained from two experiments (four wells per experimental group). The LH concentration in each well was determined using three different aliquots of the medium. The asterisk indicates the LH release is significantly higher ($p < 0.01$) than that of the other groups.

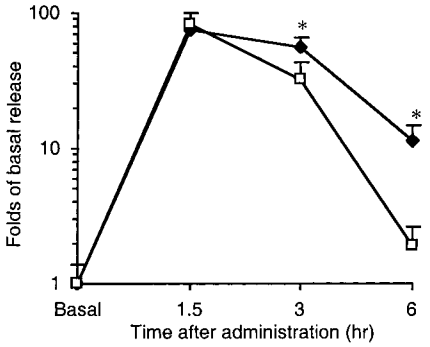

Figure 6. Induction of LH release in rats by intraperitoneal administration of [D-Lys[6](Emo)]GnRH or of the parent peptide. Rats were injected with 0.04 nmol/rat of [D-Lys[6](Emo)]GnRH (◆) or with 0.4 nmol/rat of [D-Lys[6]]GnRH (□). Blood samples were taken from each rat at the indicated time intervals, and serum LH levels were determined by RIA. Note the logarithmic scale of the *Y*-axis. The results are the mean ± SEM of LH concentrations in the serum of five animals per experimental group. The LH concentration was determined using three different dilutions of each serum sample. Similar results were obtained in two other experiments. The LH concentration in unstimulated rats was 0.53 ng/mL of serum. The asterisk indicates the LH release is significantly higher ($p < 0.01$) than that in the group treated with [D-Lys[6]]GnRH.

7 days resulted in a greater decrease in the testicular and ventral prostate gland weights as compared to those of rats that were treated with a 10-fold higher dose of the parent peptide (1 nmol/rat) (Figure 7).

As indicated earlier, emodin is known to bind to human serum albumin (HSA). To determine whether the prolonged in vivo activity of [D-Lys[6](Emo)]GnRH might be related to its binding to plasma proteins, we measured its association with HSA. As shown in Figure 8 emodic acid binds to HSA at the range of the concentrations that were examined. Consequently, the conjugation of emodic acid to [D-Lys[6]]GnRH generates a superagonist, [D-Lys[6](Emo)]GnRH, which binds significantly to HSA, and thus may explain its prolonged in vivo activity.

Emodin is known to be a photoactive compound that generates reactive oxygen species (ROS) upon illumination.[21,22] We therefore examined its cytotoxicity as well as that of [D-Lys[6](Emo)]GnRH to a mouse gonadotroph cell line (αT3-1) that expresses high-affinity binding sites for GnRH.[23] Although emodic acid demonstrated significant phototoxicity to αT3-1 cells at a concentration of 10 μM, its conjugate, [D-Lys[6](Emo)]GnRH, did not exhibit any toxicity (Figure 9). Both compounds did not show any toxicity to these cells while incubated in the dark.

To further ensure that [D-Lys[6](Emo)]GnRH is not toxic to pituitary cells, emodic acid and [D-Lys[6](Emo)]-GnRH were tested for their ability to induce an apoptotic-like process in αT3-1 cells. These experiments were carried out in dark conditions as well as under illumination. As shown in Figure 10 emodic acid and its GnRH analogue did not cause any DNA fragmentation in the dark (lanes 4 and 6), while upon illumination, only emodic acid (lane 3) but not [D-Lys[6](Emo)]GnRH (lane 5) induced DNA fragmentation.

Discussion

Development of new potent GnRH agonists or antagonists is of major interest largely because of their clinical application in modulating the reproductive system as well as in cancer therapy. It was shown by Schally et al. that incorporation of an anthraquinone moiety such as 2-(hydroxymethyl)anthraquinone hemiglutarate (Figure 1) to [D-Lys[6]]GnRH generated an agonist with bioactivity superior to that of the parent peptide.[14] This conjugate was designed to be used for targeted chemotherapy whose cytotoxicity stems from the 2-(hydroxymethyl)anthraquinone moiety.[14,16] Considering the relatively high bioactivity of [D-Lys[6](AntrQ)]-GnRH, we tried to develop an anthraquinone-based GnRH agonist possessing properties similar to those of [D-Lys[6](AntrQ)]GnRH but devoid of its toxic effect. We have previously shown[24] that incorporation of the anthraquinone-based molecule emodic acid to [D-Lys[6]]-GnRH diminished its ability to generate ROS, which probably mediate the toxicity of the antitumor anthraquinones.[25] Moreover, binding of peptides to serum proteins, such as human serum albumin, has been proposed to prolong the activity of the peptides.[10,18] Albumin, the most abundant protein in human serum (with a concentration of about 0.6 mM), possesses a half-life in circulation of 19 days. Its role in the circulatory system is probably to aid in the transport, metabolism, and distribution of exogenous and endogenous ligands.[26] Emodin is a naturally occurring polyhydroxylated anthraquinone which binds strongly to serum proteins.[17] Thus, conjugation of emodin to [D-Lys[6]]GnRH may generate a nontoxic, potent, and long-acting agonist.

In this study we have synthesized [D-Lys[6]]GnRH derivatives bearing different quinone moieties attached to the ϵ-amino side chain of Lys[6]. The one-pot synthesis in a homogeneous solution, using PyBOP as a coupling reagent, was more efficient than previously used procedures employing carbodiimides or reactive esters as coupling reagents.[13,14,19] Next we prepared [D-Lys[6]]-GnRH derivatives on a solid support, using Rink amide resin and standard Fmoc-protected amino acids and reagents (Figure 2). Using Mtt as an orthogonal protect-

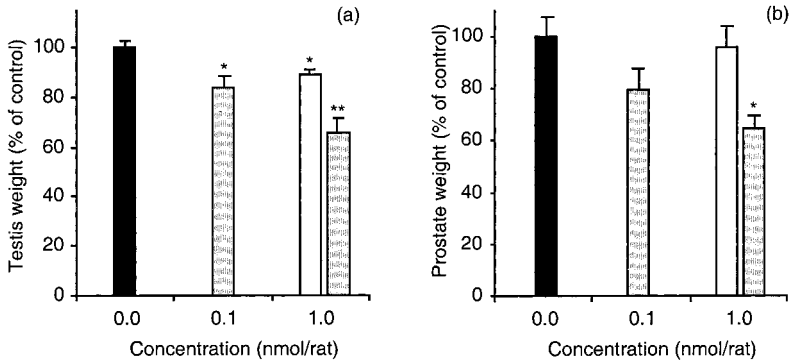

Figure 7. Effects of long-term administration of GnRH analogues on the weight of the testicular and prostate glands. Adult male rats were injected daily for 7 days either with [D-Lys^6(Emo)]GnRH (0.1 or 1 nmol/rat, gray) or with [D-Lys^6]GnRH (1 nmol/rat, white) in PBS. Control groups (black) were injected with PBS. Rats were sacrificed 24 h after the last injection; the testicular (a) and prostate (b) glands were dissected and weighed. The results are the mean ± SEM of the weight of the organs (six animals per experimental group). The weights of the testicular and prostate glands of the control group were 3.367 ± 0.07 and 0.267 ± 0.02 g, respectively. One asterisk indicates the weights are significantly different ($p < 0.01$) from those of the control. Two asterisks indicate the weights are significantly different ($p < 0.001$) from those of the control group or from those of rats treated with [D-Lys^6]GnRH (1 nmol/rat).

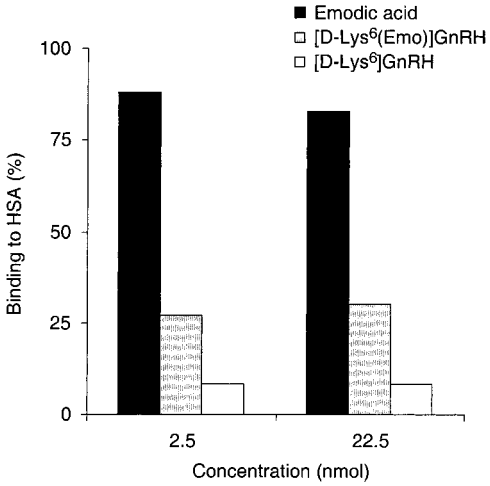

Figure 8. Binding of emodic acid and GnRH analogues to HSA. HSA (22.5 nmol; 1.5 mg in 0.5 mL of PBS) was incubated with the tested compounds at the indicated concentrations for 3 h at 37 °C. The unbound compound was then separated from the HSA by applying the mixture to a Centricon column. The HSA-bound compound was precipitated by acetonitrile, and the supernatant was removed and evaporated to dryness. The residue was dissolved in buffer A and analyzed by HPLC. The results represent the mean of two experiments.

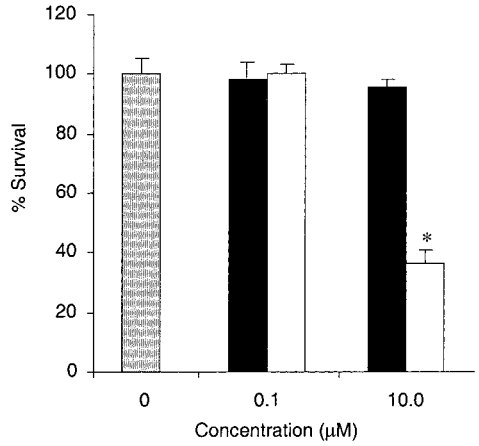

Figure 9. Phototoxicity of [D-Lys^6(Emo)]GnRH and emodic acid to αT3-1 cells. The cells were incubated for 3 h with [D-Lys^6(Emo)]GnRH (black) or emodic acid (white), washed, and illuminated. Incubation was continued for an additional 24 h, and cell survival was determined by the XTT method. Values are expressed as percent survival. Survival of 100% (gray) refers to the survival of cells in the control group that were incubated without any emodic acid derivatives. The asterisk indicates survival is significantly lower ($p < 0.001$) than that in the control group.

ing group for the N^{ϵ}-amino function of the Lys residue, we were able to unmask the N^{ϵ}-amino group without affecting removal of the other protecting groups, thus facilitating the coupling of the quinone moieties to the free amine group. In view of the convenience, better yields, and higher purity of the latter approach, it should be considered preferable for the selective synthesis of different peptide−drug chimeras that carry diverse functional groups.

In general, our studies have demonstrated a correlation between the affinities of binding to GnRH receptors and the in vitro LH-releasing potencies of the [D-Lys^6-(NQ)]GnRH and [D-Lys^6(AntrQ)]GnRH conjugates. [D-Lys^6(Emo)]GnRH, however, exhibited high LH-releasing potency despite its relatively low binding affinity (Figures 3 and 4). The gonadotropin-releasing activity of this conjugate could be completely inhibited by the antagonist [D-Pyr^1, D-Phe^2, D-Trp^{3,6}]GnRH, indicating that this activity is receptor mediated (Figure 5).

Although [D-Lys^6(AntrQ)]GnRH exhibited higher receptor binding affinity than other conjugates, the superior in vitro activity of [D-Lys^6(Emo)]GnRH prompted us to further study its in vivo biological properties. Administration of [D-Lys^6(Emo)]GnRH to rats demonstrated that this conjugate is a powerful long-acting agonist which preserves its activity significantly longer than the parent peptide (Figure 6). Moreover, chronic treatment of the adult males with the analogues showed that the conjugate is much more active in reducing the weights of the testicular and prostate glands than the parent peptide (Figure 7). Development of potent, long-acting GnRH analogues is of particular interest, since in the clinic GnRH analogues are frequently administered in slow-release depo preparations to desensitize the pituitary gland. The long-term bioactivity of [D-Lys^6-(Emo)]GnRH may be attributed, at least partly, to the high affinity of binding of the emodin moiety to serum proteins, which may protect the peptide from proteolytic

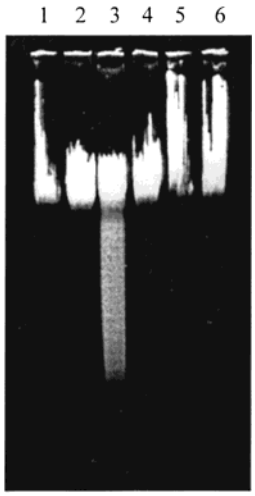

Figure 10. DNA cleavage in cells treated with [D-Lys^6(Emo)]-GnRH and emodic acid. αT3-1 cells were incubated for 5 h in darkness with the tested compounds (10 μM), washed, and illuminated. After 24 h of incubation DNA was isolated from the treated cells and analyzed by gel electrophoresis (0.4% agarose gel, ethidium bromide staining). Lanes 1, 3, and 5 represent DNA of cells treated with 1% DMSO in PBS, emodic acid, and [D-Lys^6(Emo)]GnRH, respectively, followed by illumination. Lanes 2, 4, and 6 represent cells treated with 1% DMSO in PBS, emodic acid, and [D-Lys^6(Emo)]GnRH, respectively, in darkness.

degradation. Indeed, we found that conjugation of emodic acid to [D-Lys^6]GnRH increases the affinity of binding of [D-Lys^6]GnRH to HSA (Figure 8).

As described in the Results, the in vitro LH-releasing potency of [D-Lys^6(Emo)]GnRH was superior to that of other conjugates despite its substantial lower affinity to rat pituitary GnRH receptors (Figures 3 and 4). This discrepancy might, perhaps, stem from the fact that the nature of its peptide−receptor binding may be different from that of the other derivatives, due to a combination of enhanced hydrophobicity (Table 1) and a high capacity to form hydrogen bonds through the hydroxylic groups of the emodic acid moiety. Such interactions may reduce the binding affinity but enhance receptor activation.

The assumption that the high LH-releasing activity of [D-Lys^6(Emo)]GnRH may result from its cytotoxicity and cellular damage, eventually leading to leakage of LH from the gonadotropic cells, was ruled out by evaluation of the toxicity as well as the apoptotic potency of the conjugate. These results clearly revealed that the peptide was devoid of any toxic activity. Since emodic acid is considered to be a photoactive anthraquinone, these studies were carried out in darkness as well as under illumination. Indeed, the results showed that in the dark neither emodic acid nor [D-Lys^6(Emo)]-GnRH was toxic and neither could induce apoptosis to the pituitary cell line, while upon illumination emodic acid, but not [D-Lys^6(Emo)]GnRH, was cytotoxic and induced apoptosis (Figures 9 and 10). These results are indeed supported by our previous studies, which revealed that whereas emodic acid generated ROS upon irradiation, [D-Lys^6(Emo)]GnRH was much less active in this respect.[24]

Experimental Section

All chemicals and reagents were of analytical grade. Rink amide resin, Fmoc-protected amino acid derivatives, and all the reagents for solid-phase peptide synthesis were purchased from Novabiochem (Läufelfingen, Switzerland). Side chain protecting groups employed for peptide synthesis were as follows: Arg, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf); His, trityl (Trt); D-Lys, 4-methyltrityl (Mtt) or *tert*-butyloxycarbonyl (Boc); Trp, *tert*-butyloxycarbonyl (Boc); Ser and Tyr, *tert*-butyl (tBu). Reversed-phase HPLC was performed on a Spectra-Physics SP-8800 liquid chromatography system equipped with an Applied Biosystems 757 variable-wavelength absorbance detector. HPLC prepacked columns were Lichrocart, containing Lichrosorb RP-18 (250 × 10 mm; 7 μm; Merck, Darmstadt, Germany) for semipreparative purification and Lichrospher 100 RP-18 (250 × 4 mm; 5 μm; Merck, Darmstadt, Germany) and wide-pore butyl C4 (250 × 4.6 mm; 5 μm; J. T. Baker Inc., Phillipsburg, NJ) for analytical purposes. HPLC purification and analysis were achieved by using a linear gradient established between 0.1% trifluoroacetic acid (TFA) in water as buffer A and 0.1% TFA in 75% aqueous acetonitrile as buffer B. The eluent composition was 10−100% B over 40 min, using the RP-18 column, and 0−100% B over 40 min employing the wide-pore butyl C4 column. NQ, Emo, and AntrQ were synthesized as described[14,24,27] (for the chemical structures see Figure 1).

Peptide Synthesis. Automated solid-phase peptide synthesis was performed using a multiple-peptide synthesizer (AMS-422, Abimed Analysen-Technik GmbH, Langenfeld, Germany) with Rink amide resin (25 μmol scale) as a polymeric support, following the company's protocol for the Fmoc strategy.[19,28,29] For the synthesis of [D-Lys^6]GnRH conjugates on a solid-phase support, the Mtt protecting group was used as the side chain protecting group for the D-Lys^6 residue. The completed peptide chain was cleaved from the resin, along with side chain deprotection, using 3 mL of the mixture TFA/H_2O/triethylsilane (95:2.5:2.5, v/v), for 2 h at room temperature. The crude products were precipitated with ice-cold *tert*-butyl methyl ether. Precipitated peptides were washed with cold dry *tert*-butyl methyl ether, dissolved in water or water/acetonitrile solution, and lyophilized. Peptide purification to homogeneity (usually >96%) was achieved with semipreparative HPLC and tested by analytical HPLC using the above solvent systems. Samples of each of the peptides were hydrolyzed (6 N HCl, 110 °C, 22 h, in a vacuum) and analyzed with a Dionex automatic amino acid analyzer. The results were also used for quantification of the peptide content in each preparation. The peptides were also analyzed by a Micromass Platform LCZ 4000 (Manchester, U.K.) using an electron spray ionization (ESI) technique. For biological evaluations, pure peptides were dissolved in dimethyl sulfoxide (DMSO) or in double-distilled water to obtain a 1 mM stock solution. The DMSO contents in the preparations used for bioassays were always 1% or lower. Identical concentrations of DMSO were tested and found to have no significant effect on receptor binding assays or hormone secretion.

[D-Lys^6]GnRH Conjugates. (a) Solution Synthesis. [D-Lys^6]GnRH was automatically synthesized on a multiple-peptide synthesizer and lyophilized (the purity of the crude product was >90%) as described above. To the DMF solution (1 mL) of dry crude peptide (31 mg, 25 μmol) and corresponding quinone (27.5 μmol) in the presence of NMM (8.2 μL, 75 μmol) was added a DMF solution (0.5 mL) of PyBOP (13 mg, 27.5 μmol). The mixture was stirred for 2 h at room temperature. The progress of the reaction was followed by the disappearance of [D-Lys^6]GnRH as revealed by analytical HPLC. Upon completion of the reaction the crude peptide was precipitated with ice-cold *tert*-butyl methyl ether (10 mL) and dried. Purification to homogeneity was achieved by semipreparative HPLC to yield 22.7 mg (15 μmol; 60%) of [D-Lys^6(*N*-(2-chloro-1,4-naphthoquinonyl)-β-alanine)]GnRH. Mass spectrometry: *m*/*z* found $[M + H]^+$ 1516, calcd for $C_{72}H_{92}ClN_{19}O_{16}$ $[M + H]^+$ 1516.07. Amino acid analysis after hydrolysis with 6 M HCl at 110 °C for 22 h: Glu 1.00, His 1.00, Ser 0.87, Tyr 0.98, Lys 1, Leu 0.98, Arg 1.05, Pro 1.01, Gly 0.98. Trp was destroyed under the acidic conditions of hydrolysis. In this manner, [D-Lys^6(AntrQ)]GnRH and [D-Lys^6(Emo)]GnRH were also syn-

thesized in solution (yields 65% and 57%, respectively) and characterized. Mass spectrometry: ([D-Lys^6(AntrQ)]GnRH) *m/z* found $[M + H]^+$ 1589.7, calcd for $C_{79}H_{98}N_{18}O_{18}$ $[M + H]^+$ 1588.7; ([D-Lys^6(Emo)]GnRH) *m/z* found $[M + H]^+$ 1537.6, calcd for $C_{74}H_{90}N_{18}O_{19}$ $[M + H]^+$ 1536.7. Amino acid analysis after hydrolysis with 6 M HCl at 110 °C for 22 h: ([D-Lys^6(AntrQ)]-GnRH) Glu 0.99, His 1.01, Ser 0.87, Tyr 0.98, Lys 1.02, Leu 1.02, Arg 1.05, Pro 0.61, Gly 1.06; ([D-Lys^6(Emo)]GnRH) Glu 1.01, His 0.95, Ser 0.88, Tyr 0.97, Lys 1.02, Leu 1.04, Arg 1.04, Pro 0.42, Gly 1.1.

(b) Solid-Phase Synthesis. pGlu-His(Trt)-Trp(Boc)-Ser-(tBu)-Tyr(tBu)-D-Lys(Mtt)-Leu-Arg(Pbf)-Pro-Gly-[Rink amide resin] (25 μmol), synthesized using a multiple-peptide synthesizer, was treated with 2% TFA in CH_2Cl_2 (2 mL × 5, 5 min) to cleave the Mtt group from D-Lys.[20] The removal of the Mtt group was revealed by the ninhydrin test. The resin was then neutralized by 10% *N*,*N*′-diisopropylethylamine in CH_2Cl_2 (2 mL × 3, 2 min) and washed with CH_2Cl_2 (2 mL × 3). Emodic acid (30 mg, 100 μmol) in DMF (0.1 mL) was then coupled to the free N^ϵ-amino group of D-Lys^6 following the above-mentioned company's protocol.[29] Cleavage of the resulting derivatives, precipitation, and purification were carried out as described above. Yield: 31 mg (20 μmol, 80%, based on the initial amino group−resin loading). Mass spectrometry: *m/z* found $[M + H]^+$ 1537.4, calcd for $C_{72}H_{92}ClN_{19}O_{16}$ $[M + H]^+$ 1536.7. Similar to this procedure, [D-Lys^6(AntrQ)]GnRH and [D-Lys^6(NQ)]GnRH were synthesized on a polymer support to yield 85% and 83%, respectively. Mass spectrometry and amino acid analysis yielded the expected results, similar to those obtained from the synthesis in solution.

Animals. Wistar-derived rats were obtained from the Weizmann Institute Animal Resource Center. Experiments were carried out in compliance with the regulations of the Weizmann Institute of Science.

Cells and Culture Conditions. All tissue culture components were purchased from Biological Industries (Beit Haemek, Israel). Mouse pituitary gondotrope carcinoma cell line (αT3-1) was obtained from Dr. M. Liscovitch (Department of Biological Regulation, Weizmann Institute of Science) and maintained routinely in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (complete medium). Cells were kept in a 5% CO_2 atmosphere and at 37 °C in a humidified incubator. Primary pituitary cell cultures were prepared from 21-day-old Wistar-derived female rats as described,[30] maintained in M-199 containing 10% horse serum and antibiotics, and incubated as described above. Irradiations were carried out with a 100 W halogen lamp (Philips, Germany) with an appropriate filter using a band-pass of 320−510 nm and λ_{max} = 400 nm. The fluence rate used for all the irradiations was 14.3 mW cm^{-2} with a total fluence of 17 J cm^{-2}.

GnRH Receptor Binding Assay and LH-Releasing Potency of [D-Lys^6]GnRH Derivatives. Displacement binding assays were carried out using rat pituitary membrane preparations and ^{125}I[D-Lys^6]GnRH as radioligand for 90 min at 4 °C as described.[30] Nonspecific binding was defined as the residual binding in the presence of excess [D-Lys^6]GnRH (1 μM). Specific binding was calculated by subtracting the nonspecific binding from the maximal binding, determined in the absence of any competing peptide. For evaluating the LH-releasing potencies of the [D-Lys^6]GnRH derivatives, rat pituitary cells were incubated in M-199 (without serum and antibiotics) containing the desired concentrations of the tested peptides in the dark at 37 °C for 4 h as described.[31] LH contents were analyzed by double-antibody radioimmunoassay (RIA)[32] using kits kindly supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIMDD). Results are expressed in terms of LH−RP-3 rat reference preparation.

In Vivo LH Release. Female rats (∼250 g) were intraperitonealy injected with the desired concentration of [D-Lys^6-(Emo)]GnRH or [D-Lys^6]GnRH in 0.5 mL of PBS. Blood samples were withdrawn by cardiac puncture under light ether anesthesia at the indicated time intervals, and serum LH levels were determined by RIA as described above.

Long-Term Treatment of Animals with GnRH Analogues. Intact adult males rat (260−280 g) were injected daily intraperitonealy with the GnRH analogues (0.1 or 1 nmol/rat) in 0.5 mL of PBS for 7 days. Control rats were injected daily with 0.5 mL of PBS. Rats were sacrificed 24 h after the last injection, and the organs were immediately dissected and weighed.

Binding to HSA. The binding capacity of [D-Lys^6(Emo)]-GnRH, [D-Lys^6]GnRH, and emodic acid to HSA (Sigma, St. Louis, MO) was evaluated by incubating various concentrations of the tested compounds with HSA (1.5 mg in 0.5 mL of PBS) at 37 °C for 3 h. The concentration of HSA in the solution was determined using a molar extinction coefficient of 39 000 M^{-1} cm^{-1} at 277.5 nm.[33] The unbound compound was then separated from the HSA solution by applying it to a Centricon concentrator column (Amicon Inc., Beverly, MA) with a band-pass membrane of 30 000 kD according to the manufacture's protocol. The HSA-bound compound (∼20 μL) was then precipitated by adding it to acetonitrile (1 mL). The supernatant was kept, and the precipitate was dissolved in PBS (100 μL) and reprecipitated. The combined supernatants were evaporated, and the residue was dissolved in water containing 0.1% TFA (buffer A) and analyzed by HPLC to determine the amount of the tested compounds.

In Vitro Toxicity Measurements. αT3-1 cells (50 000 cells/well) were plated in 96-well tissue culture plates in 0.1 mL of complete medium. After 24 h, the medium was changed and the cells were incubated in the same medium (without serum and phenol red) containing different concentrations of the tested compounds for 4 h at 37 °C. The cells were then washed (3×) with PBS and illuminated (λ_{max} = 400 nm) in PBS. The fluence rate was 14.3 mW cm^{-2} with a total fluence of 17 J cm^{-2}. Following illumination, the media were replaced by complete medium and the plates were incubated for an additional 24 h at 37 °C. Cell survival was determined using the XTT (Biological Industries, Beit-Haemek, Israel) kit following the manufacture's protocol.

DNA Fragmentation Assay. αT3-1 cells (5 × 10^6/dish) were incubated in phenol red- and serum-free complete medium (37 °C, 5% CO_2) with the tested compounds for 5 h in the dark. The cells were then washed with PBS (3×) and illuminated or kept in darkness as described earlier. The media were then replaced by complete medium, and the cells were incubated for an additional 24 h. DNA was then isolated, using the Wizard genomic DNA purification kit (Promega, Madison, WI) following the company's protocol, and analyzed by gel electrophoresis (0.4% cross-linked agarose).

Abbreviations

The abbreviations of common amino acids are in accordance with the recommendations of IUPAC. Additional abbreviations: AntrQ, 2-(hydroxymethyl)anthraquinone; BSA, bovine serum albumin; DMF, *N*,*N*′-dimethylformamide; DMSO, dimethyl sulfoxide; ED_{50}, concentration of ligand which indicates 50% of the maximal effect. Emo (emodic acid), 1,3,8-trihydroxy-6-carboxy-9,10-anthraquinone; GnRH, gonadotropin-releasing hormone; HPLC, high-performance liquid chromatography; IC_{50}, concentration of ligand which displaces 50% of the bound tracer; LH, luteinizing hormone; MB, maximal binding; NQ, 2-β-alanyl-1,4-naphthoquinone; PBS, phosphate-buffered saline; pGlu, pyroglutamic acid; RIA, radioimmunoassay; SEM, standard error of the mean.

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