Articles

Receptor-Mediated Targeting of a Photosensitizer by Its Conjugation to Gonadotropin-Releasing Hormone Analogues

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Photodynamic therapy uses a combination of light, oxygen, and a photosensitizer to induce the death of malignant cells. To improve the selectivity of a photosensitizer toward cancerous cells that express gonadotropin-releasing hormone (GnRH) receptors, protoporphyrin IX (PpIX) was conjugated to a GnRH agonist, [D-Lys^6]GnRH, or to a GnRH antagonist, [D-pGlu^1, D-Phe^2, D-Trp^3, D-Lys^6]GnRH. The condensation of the peptide with PpIX was carried out in a homogeneous solution using benzotriazole-1-yloxytris(pyrrolidinophosphonium) hexafluorophosphate as a coupling reagent. Although these conjugates had lower binding affinity to rat pituitary GnRH receptors than their parent analogues, they fully preserved their agonistic or antagonistic activity in vitro and in vivo. The GnRH agonist conjugate proved to be long-acting in vivo. Thus, 24 h after its administration to rats (2 nmol/rat), serum LH concentrations were significantly higher than in rats treated with the same amount of the parent peptide. The conjugates, notably the agonist, were more phototoxic toward pituitary gonadotrope αT3-1 cell line than was unconjugated PpIX. In contrast to PpIX, the phototoxicity of the conjugates toward αT3-1 cells or to human breast cancer cells (MCF-7 cells that were transfected with human GnRH receptors) was alleviated by co-incubation with the parent peptide, indicating that phototoxicity is receptor-mediated. The selectivity of the GnRH antagonist conjugate to gonadotrope cells in a primary pituitary culture was ∼10 times higher than that of the unconjugated PpIX. Thus, GnRH-based conjugates may affect cancer cells not only by acting as classic GnRH analogues to reduce the plasma levels of steroids by desensitization of the pituitary gland but also by selective photodamage of cells that express GnRH receptors.

Introduction

Photodynamic therapy (PDT) induces the death of cancerous cells through a combination of light, oxygen, and a photosensitizer. Porphyrin-based compounds, such as hematoporphyrin derivatives and 5-aminolevulinic acid-induced protoporphyrin IX (PpIX), are widely used photosensitizers in photodynamic therapy. Following irradiation, these compounds generate reactive oxygen species (ROS), mainly singlet oxygen ($^1O_2$). An ideal photosensitizer should be preferentially accumulated in tumor tissues relative to the surrounding normal cells. However, many photosensitizers are not ideal, being distributed fairly equally in both healthy and tumor cells. This causes direct damage to the healthy cells and results in post-therapeutic complications, such as skin cytotoxicity, on exposure to sunlight.[1,2]

Targeted chemotherapy has been suggested as a route to differentiate between healthy and afflicted cells, minimizing undesirable toxicity and producing high drug concentrations at selected loci. This may be achieved through conjugation of drugs to vectors that possess specific binding sites on the cancerous cells. Vectors such as monoclonal antibodies, proteins, peptides, or oligonucleotides have been used as carriers for drug targeting.[3−7] Therapy of a malignant disease may often exploit receptors that are preferentially expressed by tumor cells. Utilization of these receptors to target tumor cells is a potentially attractive approach, because it may offer the possibility of minimizing nonselective toxic effects. For example, conjugation of chlorin e_6 (Ce_6), a well-known porphyrin-based photosensitizer, to insulin or to epidermal growth factor (EGF) enhanced dramatically the corresponding photodynamic activity toward cells that express insulin or EGF receptors as compared to free Ce_6.[8,9] In addition, multiple drug resistance is expected to be less significant in the case of receptor-mediated active transport of a drug across the cell membrane.

In view of the abundance of tumors having receptors for gonadotropin-releasing hormone (GnRH), targeted chemotherapy based on GnRH analogues has gained considerable attention.[10−12] GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is a key integrator between the nervous and the endocrine systems and plays a pivotal role in the regulation of the reproductive system. The pulsatile secretion of GnRH prompts the

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anterior pituitary to release the gonadotropic hormones, luteinizing hormone (LH), and follicle stimulating hormone (FSH), which, in turn, stimulate gonadal steroidogenesis and gametogenesis.[13,14] GnRH analogues, both agonists and antagonists, have attracted great interest because of their potential application in the treatment of diseases such as prostate and breast cancer.[15−17] Their mechanism of action is believed to be, at least partially, related to gonadal steroid deprivation. This phenomenon results from the continuous action of the GnRH analogues that leads to down-regulation of the GnRH receptors and to desensitization of the pituitary gonadotropes. However, GnRH analogues have also been shown to exert direct inhibitory effects on cancer cells of prostate, breast, and ovarian cancers.[16,18−22]

It is well established that 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 or their internalization by gonadotropes.[23] Thus, several agonists and antagonists of GnRH were attached to various cytotoxic compounds, and the resulting conjugates were evaluated for their anticancer activity. The conjugates exhibited a wide range of specific binding affinities toward GnRH receptors and were also internalized by the cell.[10−12] The cytotoxicity of some peptide−drug hybrids was markedly augmented, far beyond that of the drug component. However, treatment of GnRH-related tumors by these conjugates also caused damage to normal pituitary gonadotropes, probably because of their concomitant binding to healthy cells. Although the damage to pituitary cells was reported to be reversible,[24] the use of these conjugates for treatment of malignancies should be carefully considered. We have therefore hypothesized that such side effects may be significantly minimized or even bypassed by attaching a photosensitizer to GnRH. The resulting phototoxic conjugate would be activated only upon specific illumination of the afflicted tissue. Thus, conjugation of a photosensitizer to a GnRH agonist such as [D-Lys^6]GnRH could potentially improve cancer treatment in several ways simultaneously: (i) The uptake of the photosensitizer will be mediated mainly through the GnRH receptors, leading to selective concentration of the drug in those tissues that express GnRH receptors. Because only the afflicted loci are to be irradiated, selectivity and specificity of therapy would be obtained with a minimal systemic toxicity. (ii) At the pituitary level, the conjugate may act as a GnRH analogue to desensitize the gonadotropes, reduce the secretion of gonadotropins, lower plasma concentration of steroids, and thereby inhibit the growth rate of steroid-dependent cancer cells. (iii) The GnRH moiety of the conjugate can also act directly on cancer cells to lower their proliferation rate. (iv) The multidrug resistance phenomenon, a characteristic feature in classical chemotherapy, should be minimized by the receptor-mediated active transport of the conjugate across the cell membrane.

In this study we set out to evaluate the photodynamic activity and selectivity of GnRH−PpIX conjugates that we synthesized and to examine whether their phototoxicity may be attributed to receptor-mediated endocytosis. Studies related to GnRH-targeted photodynamic therapy have never been described before. We selected

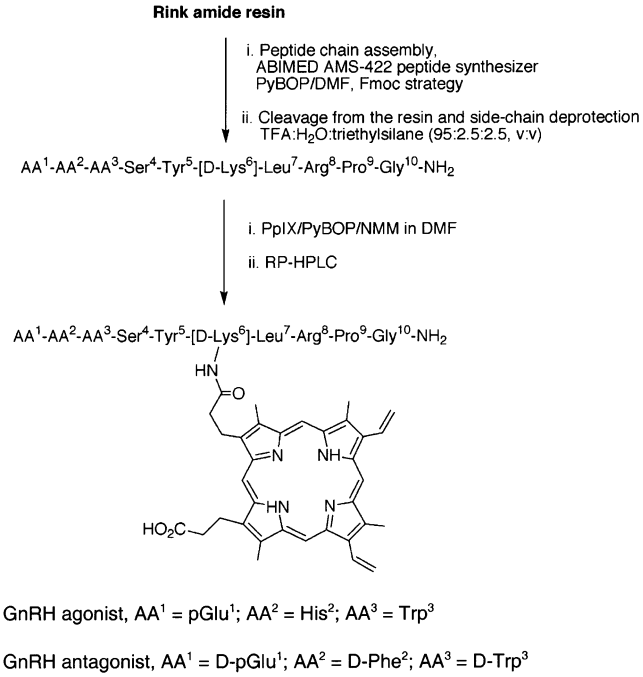

Figure 1. Scheme for the synthesis of GnRH−PpIX conjugates.

the GnRH agonist [D-Lys^6]GnRH and the GnRH antagonist [D-pGlu^1, D-Phe^2, D-Trp^3, D-Lys^6]GnRH ([D-Lys^6]-Antg) as possible vectors for targeting cancer cells because these analogues possess a free ϵ-amino group that can be conjugated to a photosensitizer. These GnRH analogues have been demonstrated to bind to high-affinity GnRH receptors[25,26] followed by internalization.[23,27] The binding affinity of these conjugates to GnRH receptors and their bioactivity were determined by using rat pituitary membranes and rat pituitary primary cultures, whereas the mouse pituitary gonadotrope cell line (αT3-1) was utilized to evaluate their phototoxicity. It has been shown that this cell line expresses GnRH receptors that are identical, structurally and functionally, to those of the rat pituitary gland.[28]

Results

Preparation of Conjugates. Parent peptides, (pGlu^1, His^2, Trp^3, Ser^4, Tyr^5, D-Lys^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH$_2$) [(D-Lys^6)GnRH] and (D-pGlu^1, D-Phe^2, D-Trp^3, Ser^4, Tyr^5, D-Lys^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH$_2$) [(D-Lys^6)Antg], were synthesized by an automatic multiple solid-phase peptide synthesizer. Conjugation of PpIX to the peptides involved the reaction of the free ϵ-amino group of the D-Lys^6 residue of the GnRH analogues with one of the carboxylic functional groups of the PpIX either in a homogeneous solution of DMF (Figure 1) or on a solid support.[29] The reaction in solution employs benzotriazole-1-yloxytris(pyrrolidinophosphonium) hexafluorophosphate (PyBOP) as a coupling reagent and 4-methylmorpholine (NMM) as a base. This "one-pot synthesis" method resulted in a better yield and purity than other methods that use carbodiimides as coupling reagents. GnRH conjugates were also prepared by employing an automatic multiple-peptide synthesizer, using Rink amide resin as the polymeric support and standard 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acid

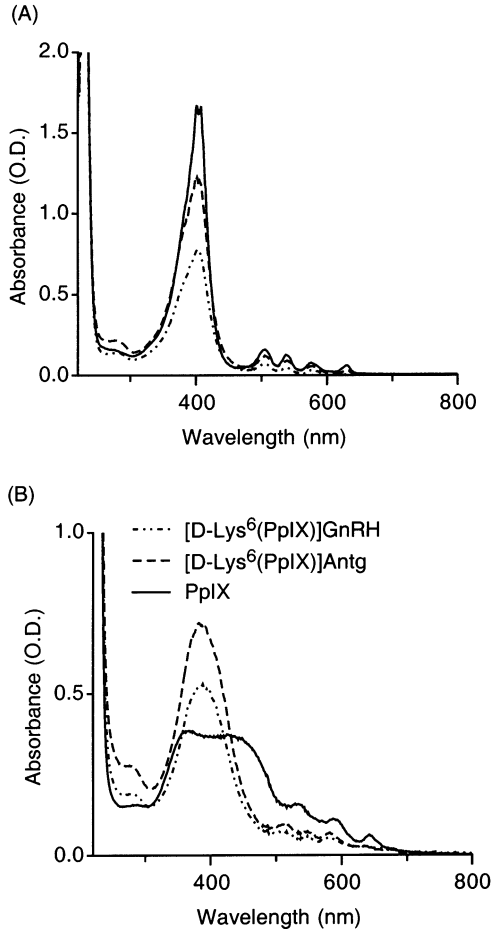

Figure 2. UV−vis spectra of PpIX and its GnRH conjugates: spectra of PpIX and its GnRH conjugates (10 μM) in ethanol (A) as compared to their spectra when dissolved in PBS (B).

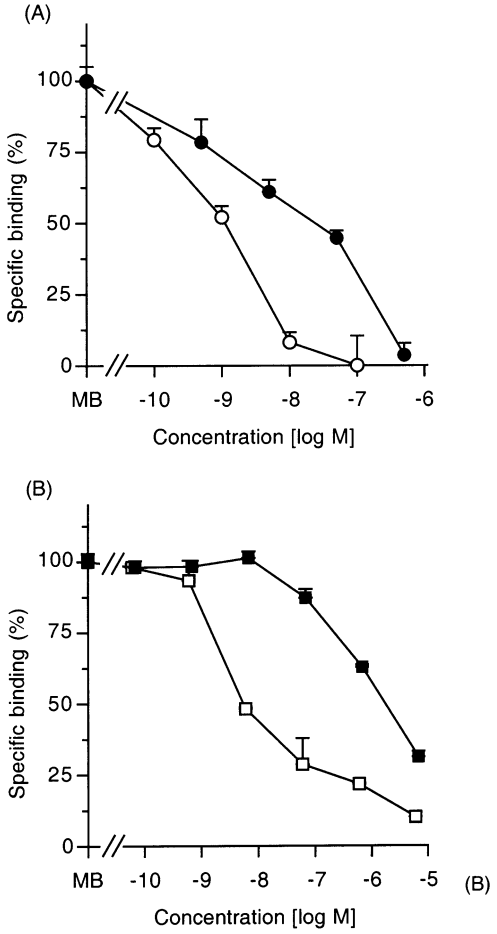

Figure 3. Displacement (percent) of specific binding of ^{125}I[D-Lys^6]GnRH from the pituitary membranes of pro-estrous rats by increasing concentrations of unlabeled GnRH analogues: (A) [D-Lys^6]GnRH (○) and [D-Lys^6(PpIX)]GnRH (●); (B) [D-Lys^6]Antg (□) and [D-Lys^6(PpIX)]Antg (■). Membranes were incubated for 90 min at 4 °C with ^{125}I[D-Lys^6]GnRH (40000−50000 cpm, 23.5 pM) either with or without the unlabeled peptides. Nonspecific binding was defined as binding of the labeled ligand in the presence of 1 μM [D-Lys^6]GnRH (A) or of 10 μM [D-Lys^6]Antg (B). These values were subtracted from the total binding for the calculation of the specific maximal binding (MB). Each result is the mean of two experiments carried out in triplicate.

as described.[29] It was found, however, that the overall yield of the GnRH conjugates was substantially higher when they were synthesized in solution (65 vs 10%). This might be due to steric effects or certain peptide−polymer association resulting in an incomplete coupling of the carboxylic function of PpIX to the ϵ-amino function of D-Lys^6. Because PpIX is not a symmetrical molecule, two isomers are theoretically expected upon such coupling. The existence of the isomers can be presumably revealed by ^{1}H NMR spectroscopy through the appearance of two distinguishable sets of resonance frequencies. However, two-dimensional NMR studies (Bruker DRX-800 spectrometer) in DMSO-d_6−H_2O did not reveal such a phenomenon. Moreover, HPLC analysis using various columns (RP-8 and RP-4) and gradients resulted only to a single peak possessing the desired mass spectra. Yet, the possible existence of two isomers cannot be excluded. The crude conjugates were then purified to homogeneity by semipreparative RP-HPLC and were further characterized by UV, MS, and amino acid analysis. Figure 2 demonstrates that PpIX is more aggregated in PBS than in ethanol, as evidenced by the flattening of its characteristic peak at 400 nm.[30] Covalent binding of PpIX to the GnRH analogues significantly reduced its aggregation state in PBS, as indicated by the sharper peaks of the conjugates compared to that of PpIX (Figure 2B).

Binding Affinities of the Conjugates toward Rat Pituitary GnRH Receptor. The ability of the GnRH conjugates to bind to GnRH receptors was evaluated in vitro by displacement assays using ^{125}I[D-Lys^6]GnRH as the radioligand. As shown in Figure 3A, the agonist-based conjugate, [D-Lys^6(PpIX)]GnRH, bound specifically to the GnRH receptor, although its binding affinity was lower than that of the parent agonist (IC_{50} = 22 and 1.1 nM, respectively). Incorporation of PpIX to the antagonist resulted in an even larger decrease in binding affinity, with an IC_{50} of 1.8 μM for [D-Lys^6-(PpIX)]Antg versus 6 nM for the parent antagonist (Figure 3B).

Biological Potencies of GnRH Conjugates. To evaluate the correlation of the binding affinity with the bioactivity, we compared the effect of each GnRH conjugate and its parent peptide on LH secretion, using primary rat pituitary cell cultures. All experiments were carried out in the dark to avoid photoactivation of the PpIX moiety, and LH secretion was determined by radioimmunoassay (RIA). The bioactivity of [D-Lys^6-(PpIX)]GnRH and of the parent peptide [D-Lys^6]GnRH

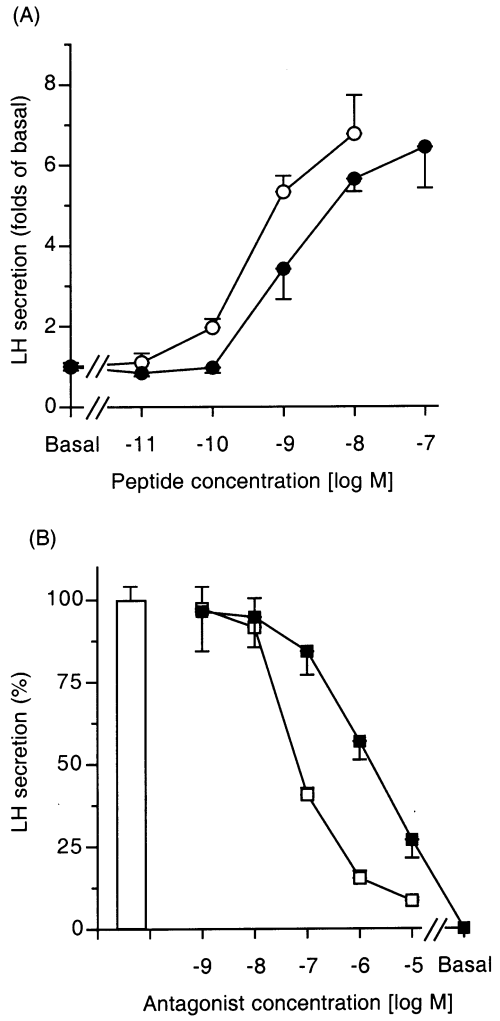

Figure 4. Effect of GnRH conjugates on the secretion of LH from primary cultures of rat pituitary cells: (A) LH releasing potency of [D-Lys^6]GnRH and of its conjugate [primary cultures of rat pituitary cells were incubated in M-199 containing the indicated concentrations of [D-Lys^6]GnRH (○) or [D-Lys^6(PpIX)]-GnRH (●)]; (B) effect of a GnRH antagonist conjugate on the inhibition of LH secretion from primary cultures of rat pituitary cells that were stimulated by GnRH [cells were incubated in the dark with GnRH (1 nM) in the absence (white bar) or presence of increasing concentrations of [D-Lys^6]Antg (□) or [D-Lys^6(PpIX)]Antg (■)]. After the incubation period (4 h at 37 °C), media were collected and LH concentration was determined by RIA. Results are the mean ± SEM of two independent experiments (four wells/experimental group, each). The basal release of LH after 4 h of incubation in M-199 was 9 ± 0.85 (ng/mL) and 26.8 ± 1.53 (ng/mL) after stimulation with 1 nM of GnRH.

(Figure 4A) demonstrated that the agonist conjugate exhibited lower LH releasing activity than its parent peptide. Similarly, we have found that the bioactivity of [D-Lys^6(PpIX)]Antg (Figure 4B) was also lower than that of its parent antagonist (ED_{50} = 1.8 and 0.08 μM, respectively). These results followed the same pattern as we found with respect to binding affinity, with the antagonist conjugate showing the lowest overall bioactivity.

We have further evaluated the agonistic activity of [D-Lys^6(PpIX)]GnRH, in vivo, by assessing the induction of LH release following intraperitoneal injection of [D-Lys^6(PpIX)]GnRH or of its parent peptide to rats. The results confirmed our in vitro findings that the parent peptide exhibits a higher bioactivity in terms of total LH release as compared to its conjugate. However, the duration of LH release induced by the conjugate was significantly longer than that induced by the parent peptide. Whereas the $t_{1/2}$ of the effect of the conjugate was ~8 h, that of the parent peptide was 2.5 h only. Thus, the serum LH levels that were induced 24 h after the administration of 2 nmol of the conjugate were significantly ($p < 0.05$) higher than those induced by an equimolar concentration of the parent peptide (0.88 vs 0.34 ng/mL). Long-lasting in vivo bioactivity was also evident when PpIX was conjugated to [D-Lys^6]Antg. Following 4.5 h of treatment of ovariectomized rats with SB-75, a potent antagonist of GnRH, the serum LH concentration dropped from 13.2 ± 0.2 ng/mL (basal release) to 6.9 ± 0.2 ng/mL, whereas at 18 h it was elevated to 10.2 ± 0.3 ng/mL. However, the serum LH concentration measured 18 h after [D-Lys^6(PpIX)]Antg administration (10.7 ± 0.2 ng/mL) was not significantly different from that determined 4.5 h following administration (10.6 ± 0.4 ng/mL).

Phototoxicity of GnRH Conjugates in Established Cell Lines. The phototoxicities of the conjugates and of unconjugated PpIX were evaluated in mouse pituitary gonadotrope cell line (αT3-1) and in a human breast cancer cell line (MCF-7). The presence of GnRH receptors in the αT3-1 is well established[31] and was confirmed using receptor-binding experiments. Phototoxicity experiments were carried out with various concentrations of the conjugates (although only the agonist conjugate was used with the MCF-7 cell line) and using two doses of illumination (8.5−17 J cm^{-2}). It was found that the optimal dose of illumination to cause phototoxicity is 17 J cm^{-2} and, therefore, the irradiation dose was set at 17 J cm^{-2} for latter experiments. Whereas the conjugates and PpIX did not exhibit any toxicity under dark conditions (data not shown), they became highly toxic to cells upon illumination with visible light (λ > 350 nm). No toxicity was apparent when untreated cells were either illuminated or kept in the dark (100% survival).

Both agonist- and antagonist-derived conjugates were more phototoxic to αT3-1 cells than the unconjugated PpIX. The LD_{50} values for [D-Lys^6(PpIX)]GnRH and [D-Lys^6(PpIX)]Antg were 95 and 100 nM, respectively, compared to 150 nM for free PpIX. Increasing the incubation time from 45 to 180 min significantly increased the phototoxicity of the conjugates to the αT3-1 cell line (Figure 5), whereas it did not affect the phototoxicity of unconjugated PpIX (Figure 5C). The greatest change was observed for [D-Lys^6(PpIX)]Antg, the phototoxicity of which increased by an order of magnitude, from LD_{50} = 1.01 μM at 45 min to LD_{50} = 0.1 μM at 180 min (Figure 5B). In contrast, in MCF-7 cells, which do not possess GnRH receptors, the phototoxicities of both [D-Lys^6(PpIX)]GnRH and PpIX were similar (LD_{50} = 180 and 200 nM, respectively).

Selective Phototoxicity of GnRH Conjugates. To determine whether the phototoxicity of the PpIX conjugates toward cells that express GnRH receptors is receptor-mediated, αT3-1 cells were treated for 45 min in the dark with increasing concentrations of the parent peptide [D-Lys^6]GnRH (1−100 nM) followed by incubation with [D-Lys^6(PpIX)]GnRH (1 μM, 3 h). If photo-

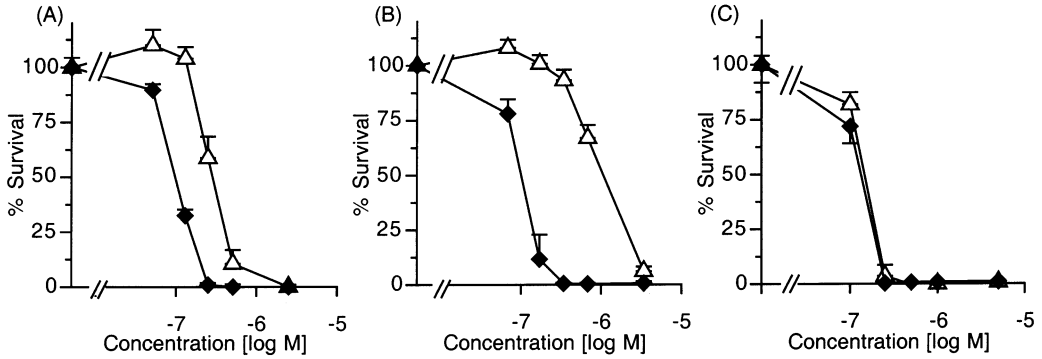

Figure 5. Time dependence of the phototoxicity of GnRH−PpIX conjugates in αT3-1 cells. Cells were incubated in the dark with [D-Lys^6(PpIX)]GnRH (A), [D-Lys^6(PpIX)]Antg (B), or PpIX (C) in phenol- and serum-free DMEM for 45 (△) or 180 min (◆). Cells were then washed and illuminated (λ > 350 nm) with an irradiance of 14.3 mW cm^{-2} and a total fluence of 17 J cm^{-2}. Incubation was then continued for 24 h in a fresh medium, and cell survival was determined by XTT. Values are expressed as percent survival. Complete survival (100%) is defined as the survival rate of cells in the control group that was incubated without any PpIX derivative and was either illuminated or kept in the dark. Results are the mean ± SEM obtained from two experiments (six wells/experimental group, each).

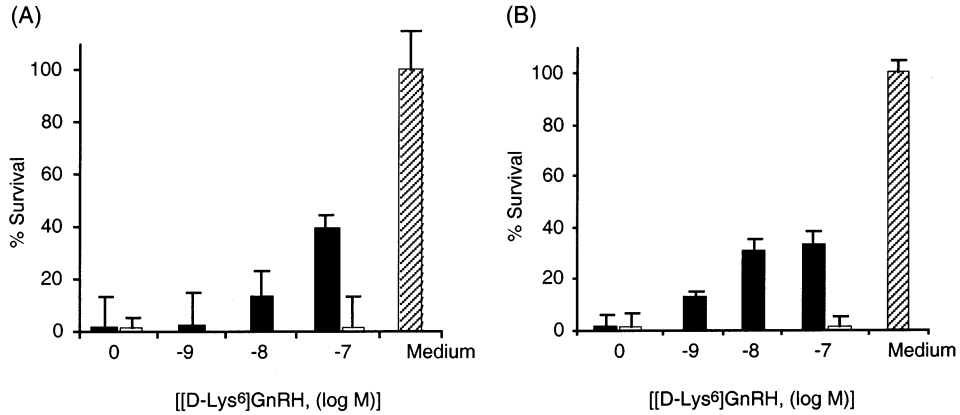

Figure 6. Reduction of the phototoxicity of [D-Lys^6(PpIX)]GnRH as a result of competition for receptor binding sites with [D-Lys^6]-GnRH. The αT3-1 pituitary cells (A) or MCF-7 cells transfected with GnRH receptors (B) were incubated for 45 min with various concentrations of [D-Lys^6]GnRH, followed by the addition of 1 μM [D-Lys^6(PpIX)]GnRH (black bars) or PpIX (white bars at the control and the 0.1 μM groups), and incubation was continued for an additional 3 h. The cells were washed, illuminated, and incubated for an additional 24 h in a fresh medium. Cell survival was determined by XTT. Complete survival (100%) was assigned to cells that were incubated in the medium only (slashed bars). Results are the mean ± SEM obtained from two experiments (five wells/experimental group, each).

toxicity is receptor-mediated, then [D-Lys^6]GnRH would be expected to compete with [D-Lys^6(PpIX)]GnRH for the same binding sites on the cell membrane and reduce the binding and the internalization of the latter and, consequently, its light-induced phototoxicity. Indeed, this proved to be the case (Figure 6A). For example, pretreatment of αT3-1 cells with 0.1 μM [D-Lys^6]GnRH resulted in an ∼40% increase in the survival rate of the pituitary cell line as compared to cells treated with [D-Lys^6(PpIX)]GnRH alone. On the other hand, the prior addition of [D-Lys^6]GnRH did not affect the phototoxicity of free PpIX. Treatment of wild-type MCF-7 cells with [D-Lys^6(PpIX)]GnRH in the presence of [D-Lys^6]GnRH did not affect phototoxicity (data not shown). However, when MCF-7 cells were transfected with plasmid DNA containing human GnRH-I receptors, results similar to those obtained for αT3-1 were observed (Figure 6B).

The selectivity of [D-Lys^6(PpIX)]Antg phototoxicity to cells that contain GnRH receptors was evaluated using primary rat pituitary cell cultures. The primary pituitary cell culture, unlike the αT3-1 cell line, is heterogeneous, containing cells that produce and release a variety of hormones, including luteinizing hormone, growth hormone, prolactin, and others. Therefore, the ratio of LH to GH concentration in the medium may indicate whether [D-Lys^6(PpIX)]Antg is preferentially phototoxic to gonadotropes that express GnRH receptors. When primary pituitary cells were treated with PpIX or [D-Lys^6(PpIX)]Antg in the dark, only basal release of LH or GH was detected. Treatment of the cells with PpIX followed by illumination brought about an increase in the content of both LH and GH in the medium, indicating that cell death and subsequent pouring out of their hormonal content is associated with illumination. However, when cells were incubated with [D-Lys^6(PpIX)]Antg for various time intervals (5−60 min) prior to illumination, the LH/GH ratios in the medium were significantly higher than those induced by PpIX, indicating a more selective damage to cells that express GnRH receptors (and contain LH) than to other pituitary cells (that contain GH). Thus, following 60 min of incubation with 1 μM [D-Lys^6(PpIX)]Antg, the LH/GH ratio was calculated to be ∼9-fold higher than that of the basal LH/GH ratio, whereas that of PpIX was 0.8-fold as compared to the basal ratio (Figure 7). However, when the time of incubation was prolonged to 3 h, the selectivity (LH/GH) of the conjugate was decreased to a level that was comparable to that of PpIX.

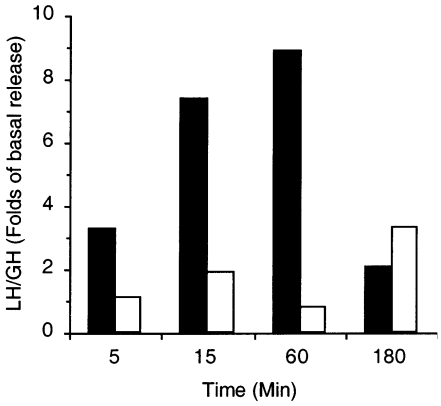

Figure 7. Selective toxicity of [D-Lys^6(PpIX)]Antg and of PpIX in primary rat pituitary cells. Cells were incubated with 1 μM [D-Lys^6(PpIX)]Antg (black bars) or with 1 μM PpIX (white bars) for the indicated time and irradiated (λ > 350 nm) with an irradiance of 14.3 mW cm^{-2} and with a total fluence of 17 J cm^{-2}. The media were then changed, and incubation was continued for an additional 24 h, after which the media were removed for the evaluation of luteinizing hormone (LH) and growth hormone (GH) concentrations by RIA. Data are reported as the folds of the basal LH/GH release (for details on calculations see Experimental Section). The fold of selectivity was calculated using the formula selectivity = $[LH_x/GH_x] \div [LH_{basal}/GH_{basal}]$, where LH_x and GH_x refer to the net amount of LH and GH poured out from pituitary cells that were incubated with either [D-Lys^6(PpIX)]Antg or PpIX and irradiated.

Discussion

Systemic toxicity and multiple-drug resistance related to chemotherapeutic agents are the most serious problems facing cancer therapy. These phenomena arise because of the lack of unambiguous, identifiable biochemical differences between normal and malignant cells. A recent modality for the treatment of hormone-sensitive prostate cancer and breast cancer is based on the use of agonists and antagonists of GnRH, which inhibit pituitary functions and consequently decrease circulatory levels of steroid hormones.[32] Breast, prostate, and other cancers have been demonstrated to express GnRH receptors, and GnRH analogues have been found to have a direct antiproliferative effect on related cancer cells.[15,18−21] Therefore, theoretically, covalent conjugation of chemotherapeutic agents to GnRH analogues may be expected to improve the efficiency of these drugs and increase their affinity and selectivity toward tumors that overexpress GnRH receptors while diminishing undesirable side effects. Indeed, GnRH analogues carrying cytotoxic drugs have been found to bind with high affinity to GnRH receptors and to be internalized;[23,25,27,33] however, because healthy pituitary gonadotropes also express GnRH receptors, these cells were also damaged.[24]

We hypothesized that the conjugation of photosensitizers to GnRH analogues may be a preferable means of selectively destroying cancerous cells, through two different actions that occur simultaneously: (i) Selective illumination of the tumor site may minimize systemic toxicity. (ii) Once the GnRH conjugates are bound to the pituitary cells, they may act as classical analogues, desensitizing the gonadotropes and thus inhibiting gonadal functions.

We have used two analogues of GnRH as carriers for the photosensitizer, protoporphyrin IX (PpIX). The agonist, [D-Lys^6]GnRH, was chosen because it can be conjugated to PpIX via the only free amino group in position 6. Moreover, [D-Lys^6]GnRH conjugates are known to bind with high affinity to GnRH receptors, to induce LH release from pituitary cells, and to be internalized by a receptor-mediated mechanism.[33] [D-Lys^6]-Antg was selected as the antagonist because, unlike most others,[26,27] it can be internalized by the cell upon binding to GnRH receptors.[23]

Upon illumination in the presence of molecular oxygen, a photosensitizer generates ROS, mainly singlet oxygen, that is highly toxic to cells.[1] The efficiency of a photosensitizer in producing singlet oxygen is believed to be partly related to the degree of its aggregation in aqueous solution.[30] When aggregated, the photoactivity of a photosensitizer is reduced because its photoexcited state is rapidly deactivated to its ground state by internal energy transfer. In this study, the PBS solution of free PpIX was found to be almost totally aggregated, as indicated by hypochromism at 400 nm (Figure 2B), in contrast to the characteristic absorption for PpIX in its monomeric form, that is, in ethanol (Figure 2A). However, the conjugation of GnRH analogues to PpIX enhanced the monomeric state of the conjugates (Figure 2B).

The capability of the conjugates to bind to rat pituitary GnRH receptors was determined by competitive binding assays using ^{125}I[D-Lys^6]GnRH as a tracer (Figure 3). The results demonstrated that the binding affinities of the conjugates were lower than those of their parent peptides, with [D-Lys^6(PpIX)]Antg having the lowest binding affinity. The reduced binding of the GnRH conjugates to GnRH receptors may be attributed to the steric effect of PpIX, which impairs ligand−receptor recognition. Investigation of in vitro LH releasing potencies, carried out in the dark, demonstrated a correlation between binding affinity to GnRH receptors and bioactivity (Figures 3 and 4). Even so, the bioactivities of the conjugates were considerably higher than might have been expected given their low binding affinities. Thus, although the conjugation of PpIX to either the agonist or the antagonist significantly decreases the binding affinity of the resulting conjugate (Figure 3), the bioactivities of the conjugate are less affected (Figure 4). It is well established, however, that occupancy of only a small portion of receptors is sufficient to elicit maximal biological activity.[34,35]

The LH releasing potency of [D-Lys^6(PpIX)]GnRH was also evaluated in vivo following its intraperitoneal administration to rats. Although [D-Lys^6(PpIX)]GnRH is a weaker agonist than its parent peptide in vitro, its agonistic activity is preserved in vivo for a longer duration ($t_{1/2}$ of 8 h vs 2.5 h for the parent peptide; Table 1). Serum LH levels measured 1.5 h after the administration of [D-Lys^6]GnRH (2 nmol/rat) were indeed ∼7 times higher than those elicited with the same dose of [D-Lys^6(PpIX)]GnRH, but 6 h later the serum levels of rats treated with the conjugate were significantly higher than in rats treated with the parent peptide. The longer half-life time of the bioactivity of the conjugate in vivo may indicate differences in the pharmacokinetics of the GnRH conjugate as compared to the parent peptide. It

Table 1. Induction of LH Secretion (Nanograms per Milliliter) in Rats Following Intraperitoneal Administration of [D-Lys^6(PpIX)]GnRH or Its Parent Peptide[a]

time (h)	[D-Lys^6]GnRH	[D-Lys^6(PpIX)]GnRH
0	0.53 ± 0.07	0.53 ± 0.07
1.5	72.50 ± 2.34**	10.64 ± 0.71
3	21.92 ± 1.11**	11.57 ± 1.59
6	2.94 ± 0.26	8.10 ±0.29*
10	1.12 ± 0.10	2.90 ± 0.36*
24	0.34 ± 0.06	0.88 ± 0.09*

[a] Rats were injected with 2 nmol/rat of [D-Lys^6]GnRH or with [D-Lys^6(PpIX)]GnRH. Blood samples were taken from each rat at the indicated time intervals, and serum LH levels were determined by RIA. Results are the mean ± SEM of LH concentrations in the serum of five rats/experimental group. LH concentrations were determined using three different dilutions of each serum sample. Similar results were obtained in two other experiments. *, significantly higher LH levels than in groups treated with [D-Lys^6]GnRH ($p < 0.05$). **, significantly higher LH levels than in groups treated with [D-Lys^6(PpIX)]GnRH ($p < 0.01$).

has been previously demonstrated that protoporphyrin derivatives bind strongly to serum proteins and generate stable complexes.[36] Thus, we have shown earlier that conjugation of [D-Lys^6]GnRH to emodin, a compound known to bind strongly to serum albumins,[37] significantly improved the binding of the peptide to human serum albumin and resulted in a prolonged in vivo activity.[29] Our findings therefore suggest that the conjugates preserve their biological activity in vivo, albeit their in vitro potencies were somewhat reduced as compared to those of their parent peptide. Furthermore, because in dark conditions the conjugates are not cytotoxic, they are able to act similarly to other GnRH agonists or antagonists that are used for cancer therapy.

Phototoxicity studies with the GnRH conjugates were first carried out on a pituitary gonadotrope cell line (αT3-1). Because this cell line expresses high-affinity receptors for GnRH,[31] it was used as a model for tumors that express these receptors. The experiments were carried out using various concentrations of conjugates, different incubation periods, and two different doses of illumination. The results suggested that the PpIX moiety retained its full phototoxicity when conjugated to GnRH.

We have also shown that the phototoxicity of the conjugates is dependent upon incubation time, with [D-Lys^6(PpIX)]Antg's phototoxicity being more dependent on incubation time than that of [D-Lys^6(PpIX)]GnRH (Figure 5). The optimal period of incubation needed for a GnRH analogue to reach binding equilibrium to GnRH receptors was demonstrated to be ∼90 min.[25] Therefore, an analogue with lower binding affinity, such as [D-Lys^6(PpIX)]Antg, may require a longer period of incubation to reach binding equilibrium. Again, this is consistent with the conjugates' phototoxicity being receptor-mediated. Because PpIX is known to accumulate in cells through mechanisms that are not receptor-mediated,[38] its phototoxicity was not dependent on incubation time.

To further demonstrate that the phototoxicity of the conjugates is receptor-mediated, we evaluated, in two model systems, their selectivity toward cells that express GnRH receptors. The first model utilized the homogeneous αT3-1 mouse pituitary and MCF-7 human breast cancer cell lines. The presence of high-affinity GnRH receptors in the αT3-1 cell line is well established[31] and was confirmed by us, using receptor-binding experiments (data not shown). In contrast, the presence of GnRH receptors in MCF-7 cells is controversial. The second model utilized a heterogeneous primary pituitary cell culture expressing a variety of receptors, including GnRH receptors.

Using the αT3-1 model, we hypothesized that if the cytotoxicity of the [D-Lys^6(PpIX)]GnRH conjugate is indeed mediated by the GnRH receptors, then its co-incubation with increasing concentrations of [D-Lys^6]-GnRH should decrease its phototoxicity due to competition for the same receptor sites in the αT3-1 cells. Indeed, co-incubation of [D-Lys^6(PpIX)]GnRH (1 μM) with increasing concentrations of [D-Lys^6]GnRH using αT3-1 cells, but not MCF-7 cells, did result in a dose-dependent increase in cell survival. In contrast, survival was not affected when PpIX was co-incubated with [D-Lys^6]GnRH using either αT3-1 cells (Figure 6A) or the MCF-7 cell line (Figure 6B). Although several studies have reported the existence of high-affinity binding sites for GnRH in MCF-7 cells,[21,39] others have failed to detect them.[40] We were able to demonstrate only low-affinity binding sites for GnRH in MCF-7 cells (data not shown). However, when this cell line was transfected with human GnRH-I receptor plasmid DNA, the phototoxicity of [D-Lys^6(PpIX)]GnRH was reduced in the presence of increasing concentrations of the parent peptide in a pattern similar to that of αT3-1 cells (compare panels A and B of Figure 6). These observations suggest that it may be possible to use gene therapy as a treatment modality for similar cancers by transfecting the cancerous cells with a GnRH receptor plasmid and employing GnRH−photosensitizer conjugates as anticancer drugs.

We exploited the heterogeneous nature of primary pituitary cell cultures in order to demonstrate the role of GnRH receptors in the cytotoxic activity of the conjugate. The gonadotropes are pituitary cells that synthesize and secrete the gonadotropic hormones and express GnRH receptors. These cells comprise a minority of the pituitary cells, whereas most of the cells express other receptors and are involved in the secretion of other hormones, such as GH or prolactin. In these experiments we used the conjugate of the GnRH antagonist that inhibits the secretion of LH and does not affect GH secretion, when incubated in dark conditions. We assumed that following illumination this antagonist conjugate will damage the cells bearing GnRH receptors and, consequently, their intracellular content of LH will be poured into the medium. Unconjugated PpIX, however, will damage evenly all pituitary cell types and therefore will discharge unselectively various hormones such as GH or LH. The quantum of GH or LH that is discharged is therefore expected to be correlated with their abundance in the pituitary gland. The selectivity of the GnRH antagonist conjugate in inducing toxicity to cells that express GnRH receptors was determined by comparing the net LH to GH ratio that was poured into the medium by the PpIX antagonist conjugate to that by free PpIX. The results of these experiments suggest that [D-Lys^6(PpIX)]Antg toxicity was ∼9-fold more selective than that of the unconjugated PpIX, because the free PpIX caused a 9-fold higher depletion of GH than [D-Lys^6(PpIX)]Antg (Figure 7). The selectiv-

ity of the conjugate to induce specific damage to the gonadotropes is observed following incubations of up to 60 min, which is sufficient time to allow the conjugate to bind to the GnRH receptors and to be internalized. The selectivity of this conjugate toward gonadotropes was reduced considerably following longer incubation periods and was diminished after 3 h of incubation. Long periods of incubation with [D-Lys^6(PpIX)]Antg may lead to its higher accumulation in other cell types and damage them through mechanisms that do not involve the GnRH receptor.

Experimental Section

Abbreviations. Abbreviations of common amino acids are in accordance with the recommendations of IUPAC. BSA, bovine serum albumin; GnRH, gonadotropin-releasing hormone, (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2); [D-Lys^6]Antg, GnRH antagonist, [D-pGlu^1, D-Phe^2, d-Trp^3, D-Lys^6]GnRH; DMF, *N*,*N*′-dimethylformamide; DMSO, dimethyl sulfoxide; ED_{50}, concentration of ligand that indicates 50% of maximal effect; FCS, fetal calf serum; GH, growth hormone; HPLC, high-performance liquid chromatography; IC_{50}, concentration of ligand that displaces 50% of bound tracer; LD_{50}, concentration of substance required for the induction of the death of 50% of cells upon illumination; LH, luteinizing hormone; MB, maximal binding; PBS, phosphate-buffered saline; PDT, photodynamic therapy; pGlu, pyroglutamic acid; PpIX, protoporphyrin IX; RIA, radioimmunoassay.

All chemicals and reagents were of analytical grade. Rink amide resin, 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acid derivatives, and all of the reagents for solid-phase peptide synthesis were purchased from Novabiochem (Läufelfingen, Switzerland). Protoporphyrin IX (PpIX) and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Mass spectrometry was carried out on an API Q-STAR *plusar i*Electrospray-TOF using a MALDI source (MDS-Sciex, Applied Biosystem, Concord, ON, Canada). For amino acid composition analysis, peptides were first hydrolyzed in 6 N HCl at 100 °C for 22 h under vacuum, and the hydrolysates were analyzed with an AccQ.Tag amino acid analysis method, using a precolumn derivatization technique (Waters 2690, Milford, MA). Reversed phase HPLC was performed on a Spectra series P200 liquid chromatography system equipped with a Spectra series UV100 variable-wavelength absorbance detector (Thermo Separation Products, Riviera Beach, FL). HPLC prepacked columns used were as follows: Vydac RP-4 (250 × 22 mm; 10 μm, Vydac, Hesperia, CA) for preparative purification and Vydac RP-4 (250 × 3.2 mm; 5 μm) or Lichrospher 100 RP-8 (250 × 4 mm; 5 μm, Merck, Darmstadt, Germany) for analytical purposes. HPLC purification and analyses were achieved by using 0.1% trifluoroacetic acid (TFA) in water as buffer A and 0.1% TFA in 75% acetonitrile in water (v/v) as buffer B. Eluent composition was 10−100% B over 40 min using the RP-4 column or 40−100% B over 30 min followed by 100% B for 10 min using the RP-8 column. Irradiations were carried out with a 100 W halogen lamp (Philips, Hamburg, Germany) with an appropriate cutoff filter (λ > 350 nm). Light intensity was measured before every experiment using a power meter (Ophir Optronics, Jerusalem, Israel). The fluent rate for all if the irradiations was calculated to be 14.3 mW cm^{-2}.

Synthesis of GnRH Conjugates. Parent GnRH analogues were automatically synthesized on a multiple solid-phase peptide synthesizer as described.[41] The purity of the crude peptides was usually >90%, and therefore they were used for the next stage without further purification. To a DMF solution (1 mL) of crude [D-Lys^6]GnRH (31 mg, 25 μmol) and protoporphyrin IX (15.5 mg, 27.5 μmol) we added a DMF solution (0.5 mL) containing benzotriazol-1-yloxytris(pyrrolidinophosphonium) hexafluorophosphate (PyBOP; 13 mg, 27.5 μmol) and 4-methylmorpholine (NMM; 8.2 μL, 75 μmol). The mixture was stirred for 2 h at room temperature. The completion of the reaction was determined by the disappearance of [D-Lys^6]-GnRH as revealed by analytical HPLC. The crude conjugate was then precipitated with ice-cold *tert*-butyl methyl ether (10 mL), dried, and purified to homogeneity (>95%) by preparative HPLC to yield 27 mg (15 μmol; 60%) of [D-Lys^6(PpIX)]GnRH. Mass spectrometry results: found *m*/*z* $[M + H]^+ = 1797.8592$; calcd *m*/*z* for $C_{93}H_{116}N_{22}O_{16}$ $[M + H]^+ = 1797.9019$. Amino acid analysis results after hydrolysis with 6 N HCl at 110 °C for 22 h: Glu 1.02, His 0.99, Ser 0.90, Tyr 0.96, Lys 1.02, Leu 0.98, Arg 0.99, Pro 0.77, Gly 1.13. Trp could not be detected because of its destruction under the acidic conditions of hydrolysis. The antagonist conjugate was synthesized and characterized according to the same method but with crude antagonist used in place of crude agonist and yielding 21 mg of [D-Lys^6(PpIX)]Antg (13.3 μmol, 53%). Mass spectrometry results: found *m*/*z* $[M + H]^+ = 1807.8601$; calcd *m*/*z* for $C_{96}H_{118}N_{20}O_{16}$ $[M + H]^+ = 1807.9114$. Amino acid analysis results after hydrolysis with 6 M HCl at 110 °C for 22 h: Glu 0.97, Phe 1.19, Ser 0.8, Tyr 0.76, Lys 0.98, Leu 0.96, Arg 1.02, Pro 0.98, Gly 1.04.

Animals. Wistar-derived rats were obtained from the animal resource center of the Weizmann Institute of Science. Experiments were carried out in compliance with the regulations of the Weizmann Institute of Science.

Cells and Culture Conditions. Tissue culture components were purchased from Biological Industries (Beit Haemek, Israel). Mouse pituitary gonadotrope cell line (αT3-1)[42] and human breast cancer cell line (MCF-7) were obtained from Dr. M. Liscovitch (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 at 37 °C in a humidified incubator with 5% CO_2 atmosphere. Primary pituitary cell cultures were prepared from 21-day-old female Wistar-derived rats as described[43] and maintained in M-199 containing 10% horse serum and antibiotics. For biological evaluations, peptides were dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of 1 mM that was diluted in the medium to obtain the desired concentrations. The DMSO concentrations in preparations that were used for bioassays were always <1%. Identical concentrations of DMSO were used as control groups and were found to have no significant effects either on receptor binding or on hormone secretion.

Receptor Transfection. Subconfluent MCF-7 cells grown in 100 mm culture dishes were transfected with 2.5 μg of human GnRH-I receptor plasmid DNA (kindly provided by Dr. Z. Naor, Tel Aviv University, Israel) using the calcium phosphate method.[44] Each experiment included a transfection with a positive control reporter plasmid containing the cytomegalovirus (CMV) promoter to permit estimation of transfection efficiencies and comparison with results obtained from other experiments. One day after transfection, cells were plated in 96 well plates and cultured for an additional 24 h before the experiment was carried out.

Receptor Binding Assay. [D-Lys^6]GnRH was iodinated by the chloroamine T method and purified by analytical HPLC. The binding affinities of the GnRH analogues to preparations of rat pituitary receptors were compared through competitive binding experiments.[45] Briefly, rat pituitary membranes containing ~0.1 pituitary equiv (25 μg of protein/tube, prepared from proestrous rats) in 0.4 mL of assay buffer (0.1% BSA in 10 mM Tris-HCl; pH 7.4) were incubated in triplicate with ^{125}I[D-Lys^6]GnRH (40000−50000 cpm, 23.5 pM, 50 μL), in the presence (10 μM−0.1 nM) or absence of a GnRH conjugate (50 μL) in a total volume of 0.5 mL of assay buffer for 90 min at 4 °C. The reaction was terminated by rapid filtration through Whatman GF/C filters, presoaked in 2−3% polyethylenimine solution in 10 mM Tris-HCl to minimize filter absorption. The filters were washed three times with cold 10 mM Tris-HCl (3 mL, each) and were counted in a Packard Auto-Gamma counting system (Packard, Meriden, CT). Nonspecific binding was defined as the residual radioactivity in the presence of an excess of [D-Lys^6]GnRH (1 μM) or [D-Lys^6]Antg (10 μM).

Specific binding was calculated by subtracting the nonspecific binding from the maximal binding, determined in the absence of any competing peptide.

Effects of GnRH Conjugates on LH Release. The LH-releasing potencies of the GnRH conjugates and their precursor peptides were evaluated in primary pituitary cell cultures prepared from 21-day-old Wistar-derived female rats as described.[46] The cultures were maintained in M-199 supplemented with 10% horse serum and antibiotics and were plated (50000 cells/well) in 96-multiwell tissue culture dishes. After 48 h, the medium was replaced by phenol red and serum-free M-199 containing the desired concentration of the peptides (four wells/experimental group) and incubated in the dark at 37 °C for 4 h. To evaluate the activity of the antagonistic conjugate, primary cultures of rat pituitary cells were incubated with native GnRH (1 nM) alone and in the presence of increasing concentrations of either the conjugate or the parent antagonist peptide. All experiments were carried out in the dark to avoid photoactivation of the PpIX moiety. At the end of the incubation period, supernatants (0.1 mL) were diluted with 1% BSA/PBS (0.9 mL) and analyzed for LH and GH content by double-antibody RIA[47,48] using kits kindly supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIMDD). LH and GH levels are expressed in terms of the LH-RP-3 and GH-RP-2 rat reference preparation, respectively.

Selectivity of GnRH Conjugate to Rat Pituitary Cells. Rat pituitary cells were prepared as described earlier and were maintained in DMEM containing 10% horse serum and antibiotics. Cells were then incubated for the indicated time in the dark with the tested compounds (1 μM) in phenol-free and serum-free DMEM at 37 °C. Following irradiation at an intensity of 14.3 mW cm^{-2} for 20 min (total fluence of 17 J cm^{-2}), the medium was replaced with fresh DMEM containing serum and antibiotics, and incubation was continued in a humidified incubator at 37 °C for an additional 24 h. The supernatants (0.1 mL) were diluted with 1% BSA/PBS (0.9 mL), and LH and GH concentrations were determined by RIA. The fold of selectivity (LH/GH) was calculated using the formula

$$\text{selectivity} = \frac{[LH_x/GH_x]}{[LH_{basal}/GH_{basal}]}$$

where LH_x and GH_x refer to the net amount of LH and GH poured from pituitary cells following irradiation of either [D-Lys^6(PpIX)]Antg or unconjugated PpIX. For example, following treatment of primary pituitary cells with 1 μM [D-Lys^6(PpIX)]Antg for 60 min, the cells were irradiated for 20 min, the medium was collected 24 h later, and LH concentration was determined as 54.5 ± 5.3 ng/mL. The basal release of LH (LH_{basal}) from untreated cells irradiated for 20 min and incubated for 24 h was determined as 20.5 ± 2.1 ng/mL. Therefore, $LH_x = 34$ (54.5−20.5). Similarly, the basal GH release (GH_{basal}) from irradiated untreated cells was 88.1 ± 8.4 ng/mL, and following treatment with [D-Lys^6(PpIX)]Antg and irradiation, GH concentration was increased to 104.8 ± 3.2 ng/mL ($GH_x = 16.7$). Thus, the selectivity in this case is 8.75.

In Vivo Studies. Fifteen-week-old female rats (220−250 g) were injected intraperitonealy with the desired concentration of [D-Lys^6(PpIX)]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.

Phototoxicity Assay. αT3-1 cells (50000 cells/well) were plated in 96-well tissue culture plates in 0.1 mL of DMEM supplemented with 10% FCS and antibiotics (complete medium). After 24 h, the medium was replaced with fresh medium (but without phenol red and without FCS) containing various concentrations of the tested compounds, and cells were incubated at 37 °C for the indicated time. The medium was then removed, and cells were washed with PBS and irradiated in PBS for different periods of time to activate the PpIX moiety. The intensity of the light source in all experiments was 14.3 mW cm^{-2}, with a total fluence of 8.5 or 17 J cm^{-2}. Following illumination, the medium was replaced by DMEM supplemented with 10% FCS and plates were incubated for an additional 24 h at 37 °C. Cell survival was then determined using the XTT kit (Biological Industries, Beit-Haemek, Israel) following the manufacturer's protocol.

Statistical Analysis. Results are expressed as the mean ± SEM. Comparisons were made using unpaired *t* test; $p < 0.05$ was considered to be significant.

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