Lipoamino acid-modified GnRH analogs with receptor-mediated antiproliferative activity in prostate and ovarian cancer cells

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Abstract
Gonadotropin-releasing hormone (GnRH) analogs (e.g., triptorelin) are developed to treat hormone-dependent reproductive cancers. However, these analogs lack a significant direct antitumor activity to make them suitable for hormone-refractory reproductive cancers. In this study, we examined different biological properties of lipid-modified GnRH analogs, with/without D-amino acid substitution at position 6 in prostate and ovarian cancer cells. We revealed that the improved metabolic stability due to lipid-modification and D-amino acid substitution played a pivotal role in enhancing GnRH receptor-mediated direct antiproliferative activity up to 4.5-fold higher than triptorelin. Furthermore, a comparable FSH release and higher LH release activity in pituitary cells than triptorelin was observed, indicating an improved specificity and/or binding affinity to GnRH receptors. We confirmed the important role of sex steroids in the antitumor activity of the lipopeptides, which were contrasting in prostate and ovarian cancer cells. The superior activity of these GnRH analogs over commercial peptides renders promises for developing new GnRH receptor ligands to treat hormone-dependent and-refractory cancers, as well as emerging new targeting moieties for the delivery of anticancer agents in GnRH receptor-overexpressing cancers.

Graphical Abstract

Keywords GonRH analogs · Cancer therapy · Peptide delivery · Reproductive cancer · Lipopeptide

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Introduction

The primary regulatory component of the reproductive system is a gonadotropin-releasing hormone (GnRH), also known as luteinizing hormone-releasing hormone (LHRH). It is a decapetide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) controlling the reproductive axis and produced by neurons located in cell bodies of the hypothalamic–preoptic region [1, 2]. Following transport to the anterior pituitary gland, GnRH acts on the membrane-bound receptors on gonadotrophs to stimulate the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion [3]. LH stimulates the secretion of testosterone by the testicular Leydig cells, while FSH activates spermatogenesis and also the development of inhibin in Sertoli cells [4].

Two main groups of GnRH analogs are agonists and antagonists. The ability of GnRH agonists to induce gonadotrophic output is followed by a suppression of pituitary-gonadal function [5]. Agonists induce an increase in the secretion of LH and FSH when injected, and chronic administration induces inhibitory effects. The continuous administration of GnRH leads to the inhibition of the hypothyseal–gonadal axis via downregulation, desensitization, and consequent suppression of GnRH receptors in the pituitary [6]. It suppresses the secretion levels of LH, FSH, and sex steroids through a reversible medical castration process. The efficacy of GnRH agonists in the treatment of sex hormone-dependent malignancy depends on the level of inhibition they induce in the secretion of sex steroid hormones. The resultant sex steroid deprivation state caused by the downregulation of GnRH receptors is the basis for a variety of clinical applications of GnRH agonists like hormone-sensitive cancers of the prostate and ovary [6].

It has been shown that the reported direct antiproliferative effects of GnRH analogs are independent of their actions in decreasing sex steroid hormones [7, 8]. It has become increasingly clear that GnRH receptors are overexpressed in cancer tissues related (e.g., prostate, ovarian) [9] or unrelated (e.g., colon, lung, liver) to the reproductive system [10]. While common receptors are involved, different signaling mechanisms are direct the antiproliferative effects of GnRH analogs in cancer cells compared to the pituitary gonadotrophs [11]. More specifically, at the cancer cell level, the GnRH receptor activates a phosphotyrosine phosphatase (PTP), which counteracts with the mitogenic signal transduction of growth factor receptors, resulting in an antiproliferative activity [11]. In later stages of hormone-dependent cancers, such as hormone-dependent prostate and ovarian cancers, tumors often progress towards more aggressive and hormone-refractory mode [12, 13]. Chemotherapy, either alone or in combination with antiandrogens/antiestrogen therapy or immunotherapy in recent years, are among approaches of treating hormone-refractory tumors [14]. These treatments, however, are associated with challenges including severe side effects, inherent or acquired resistance to therapy or low response rate [15]. While these hormone-refractory tumors are no longer responsive to the sex hormone suppression therapy by GnRH agonists and antagonists, they still overexpress GnRH receptors. A strong antiproliferative, proapoptotic, antimetastatic, and antiangiogenic activity that is mediated by the Gαi/cAMP signaling pathway has been observed by GnRH agonists and antagonists [16]. Hence, these agents are now considered effective therapeutics in hormone-refractory prostate and ovarian cancers when used in combination with standard therapy. Several clinical trials have shown their efficacy in improving disease-free survival in patients [12, 15, 17]. These effects have been shown to be directly dependent on the level of GnRH receptor expression in particular tumor cells [18]. These new findings warrant the development of GnRH receptor ligands with stronger direct antitumor activity.

Furthermore, the overexpression pattern of the GnRH receptors in many cancers makes GnRH analogs attractive, targeting ligands for the delivery of therapeutic and diagnostic agents exclusively to tumor cells [19]. Several GnRH receptor-targeted delivery systems have been developed using different derivatives of GnRH peptide [20], and a few reached clinical trials [21–23]. However, those delivery systems all failed in different phases of the trial, plausibly due to the lack of stability of the targeting GnRH derivative. Lipophilic moieties coupled to peptides conferred the characteristics essential for protecting a usually labile peptide from enzymatic degradation [24]. The increased stability could considerably delay the dissociation rate of the GnRH analog from its receptor and enhance its potency in vivo. Modifications such as the addition of lipidic moieties or cyclization have been attempted to not only increase stability but also to allow transport across biological systems [25]. An established method for increasing the lipophilicity of peptides is their conjugation to lipoamino acids—alpha-amino acids with varying lengths of an alkyl side chain [26].

We previously showed that lipid-modified GnRH analogs showed significantly improved metabolic stability and membrane permeability in different in vitro models [27]. In this study, we further investigated whether this increased stability and permeability of the lipid-modified GnRH analogs would lead to a better direct antitumor activity and gonadotropin release. This might offer extra benefit for application in hormone-dependent cancers and make the new agonist(s) in addition to applications in hormone-resistant cancers of, e.g., prostate and ovary. Such analogs with improved stability will also be suitable to be used as a
targeting moiety in different drug delivery systems. The GnRH agonists are proposed to inhibit tumor growth by suppressing testosterone secretion through the endocrine system as well as exerting a direct antiproliferative action on the tumor cells. Six lipidic derivatives of the GnRH peptide (1–6) that had previously shown improved stability against enzymatic degradation and permeability across a Caco-2 cell monolayer were selected [27]. The twelve-carbon chain lipo amino acid (C12: 2-Amino-D, L-dodecanoic acid) was conjugated to either terminus of the peptide. In some analogs (compounds 1–2), the modification was performed to the N-terminus of the lipopeptides, while in others, the lipo amino acid was incorporated into the middle of the sequence in place of Leu7 (compounds 3 and 5) or to the C-terminus (compound 6). D-Tryptophan (D-Trp6 or w6) substitution was performed to produce [w6] GnRH-based derivatives. In compounds 1–2, glutamic acid (Glu) was replaced by Glutamine (Gln) as the first amino acid in the GnRH sequence. The rationale behind this modification was firstly due to a spontaneous cyclization of pyroglutamic acid in endogenous GnRH to produce N-terminal glutamine, and secondly, according to our previous findings, GnRH conjugates with Gln have longer half-lives than those with Glu [28].

Constructs 1–6 were tested in vitro for direct antiproliferative activity in human prostate and ovarian cancer cell lines. The toxicity of these ligands was examined against normal blood and pituitary cells, as well as their ability to stimulate the release of pituitary gonadotropins. Moreover, the impact of sex steroids (dihydrotestosterone, DHT, or 17ß-estradiol, E2) on the direct antitumor activities of the GnRH analogs was investigated.

### Results and discussion

We previously showed that our developed lipid-modified GnRH analogs exhibited higher metabolic stability than the currently available agonist triptorelin and the natural GnRH receptor ligand (Table 1). Improving the stability of the GnRH peptide will enhance its duration of the activity, which consequently reduces its dosing frequency. While the currently available GnRH analogs in clinical use achieve the desired pharmacological effect by their primary activity on the pituitary and through the hypothalamic–pituitary–gonadal axis (HPG axis), they do not offer significant direct antiproliferative effects against tumor cells [29]. In this research, we hypothesized that improving the stability and potentially the potency of the GnRH agonists through conjugation of lipid chains might lead to an increase in their direct antiproliferative activity [30]. This would result in the development of GnRH receptor ligands with dual action in hormone-dependent cancers, both through blocking the release of sex hormones as well as directly inhibiting the growth of the tumor. If a strong antitumor activity is achieved, they can also be potentially used in hormone-independent reproductive cancers. Herein, we investigated the biological activities of two groups of GnRH derivatives.

### In vitro antiproliferative activity studies

While GnRH receptor ligands have been shown to play a role in cell growth, invasion, and angiogenesis of different tumors [31], the dose by which they exert these activities varies GnRH receptor-positive peripheral tissues [32]. At low (nanomolar) concentrations, GnRH ligands increase cell proliferation while they inhibit cell proliferation at high (micromolar) concentrations [33]. This phenomenon has been shown to be due to the differential expression profiles of GnRH receptors [32]. In this study, tumor cell lines at different GnRH receptor expression and hormone dependence levels were used. The aim was to investigate the antiproliferative effect of the lipid-conjugated GnRH derivatives in three GnRH-receptor positive prostate cancer cell lines (LNCaP, DU145, and PC3), a high-GnRH receptor-expressing (OVCAR-3) as well as a low GnRH receptor-expressing (SKOV-3) cell lines.

The antiproliferative effect of GnRH analogs and control peptides were reported as IC50 (µM) values (Table 2). Triptorelin (shown as [w6]GnRH) was used as the parent peptide (control) for [w6]GnRH-based derivatives 2, 3, and 6, and GnRH was used as a control for GnRH-based compounds 1, 4, and 5.

### Prostate cancer cell lines

In the GnRH-based group, the proliferation of hormone-resistant DU145 was improved by 25% (IC50 = 75 µM) when these cells were incubated with compound 4 while compound 1 and 5 in this group did not reduce the growth of DU145 (IC50 > 100, Table 2). In the [w6]GnRH-based

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**Table 1** Amino acid sequence of native GnRH, commercially available triptorelin, and the lipoamino acid derivatives 1–6

| Peptide name | Amino acid sequence | t1/2 [min] [27] |
|-------------|---------------------|-----------------|
| 1 C12[Q7]GnRH | C12a QHWSYGLRPG | 48.5 ± 7.1 |
| 2 C12[Q7][w6]GnRH | C12a QHWSYwLRPG | 225.4 ± 10.4 |
| 3 [w6][C127]GnRH | pEHWSYwC12aRPG | 167.8 ± 12.5 |
| 4 [C126]GnRH | pEHWSYC12aLRPG | 79.5 ± 8.7 |
| 5 [C127]GnRH | pEHWSYGC12aRPG | 82.6 ± 9.6 |
| 6 [w6]GnRH-C12 | pEHWSYwLRPGC12a | 146.9 ± 10.1 |

GnRH (Triptorelin) | pEHWSYGLRPG | 6.4 ± 2.3 |

[w6]GnRH (triptorelin) | pEHWSYwLRPG | 110.1 ± 15.9 |

*2C12-lipo amino acid
Table 2  Antiproliferative activity of GnRH analogs in different cell line

| Lipo-GnRH Analogs | 1      | 2      | 3      | 4      | 5      | 6      | GnRH | [w6]GnRH (triptorelin) |
|-------------------|--------|--------|--------|--------|--------|--------|------|------------------------|
| DU145 IC50 (µM)   | >100   | 58.3 ± 2.2 | 28.9 ± 1.9* | 75.4 ± 4.2 | >100   | 33.4 ± 1.1* | >100 | 62.1 ± 2.1             |
| LNCap IC50 (µM)   | >100   | 58.9 ± 3.5 | 29.1 ± 1.0* | 91.6 ± 3.4 | 71.6 ± 3.1 | 34.3 ± 2.7* | >100 | 73.4 ± 3.6             |
| OVCAR-3 IC50 (µM) | >100   | >100   | >100   | >100   | >100   | >100   | >100 | 67.7 ± 3.1             |
| SKOV-3 IC50 (µM)  | >100   | >100   | >100   | >100   | >100   | >100   | >100 | >100                   |

Concentration-response curves (nonlinear regression) were used to calculate the IC50 values (µM). Data are expressed as mean ± SD from at least three independent experiments, each in triplicate. Two-way ANOVA was performed to estimate the significance of the results (*p < 0.05, the IC50 for each compared with that of their corresponding parent peptide for the same cell line).

In hormone-dependent ovarian cancer cell line (OVCAR-3), all GnRH analogs except for compounds 1 and 2 showed a significantly enhanced antiproliferative effect with IC50 values lower than their parent peptides. Compound 3 with an IC50 value of 16 µM showed the highest growth inhibition in OVCAR-3 compared to its parent peptide [w6]GnRH and other GnRH analogs (Table 2). A very similar growth inhibitory pattern was observed in hormone-dependent prostate cancer cell line LNCaP with the exception of compound 5 from the GnRH-based group. This compound showed about 24% higher growth inhibition than its parent peptide, GnRH, in these cells. Compounds 3 and 6 showed 2.5 and 2.1 times higher inhibitory effects on the proliferation of those cells compared to [w6]GnRH (p < 0.05).

Ovarian cancer cell lines

In hormone-dependent ovarian cancer cell line (OVCAR-3), all GnRH analogs except for compounds 1 and 2 showed a significantly enhanced antiproliferative effect with IC50 values lower than their parent peptides. Compound 3 with an IC50 value of 29 and 33 µM, respectively, showed the highest growth inhibition in DU145 compared to their control peptide [w6]GnRH, and other GnRH analogs (Table 2). A very similar growth inhibitory pattern was observed in hormone-dependent prostate cancer cell line LNCaP with the exception of compound 5 from the GnRH-based group. This compound showed about 24% higher growth inhibition than its parent peptide, GnRH, in these cells. Compounds 3 and 6 showed 2.5 and 2.1 times higher inhibitory effects on the proliferation of those cells compared to [w6]GnRH (p < 0.05).

Effect of GnRH glycolipids on normal peripheral blood mononuclear cells (PBMCs) and rat pituitary cells

GnRH analogs were also tested on the proliferation of noncancerous cells. Human PBMCs and isolated rat pituitary cells were used to show if the antiproliferative activity of the compounds affects these normal cells. Pituitary cells were of particular importance since GnRH receptors are mainly expressed in these cells. None of the GnRH compounds and peptide controls affected PBMC (Fig. 2A) and rat pituitary (Fig. 2B) cell proliferation in an MTT assay performed in the same way as for the tumor cell lines. The lack of adverse effects exerted by GnRH receptor ligands has been previously confirmed in different studies where the GnRH analog has been used as a targeting agent [35]. This lack of adverse effect on the pituitary cells could be explained by differentiated signaling pathways that are activated following attachment of GnRH ligands to the pituitary cells compared to cancer cells. At the pituitary level, GnRH acts via Gq/11 subunit of G protein-coupled receptors (GPCRs), causing an inositol trisphosphate (IP3)-mediated mobilization of Ca2+ as one of the known pathways to mediate GnRH-stimulated gonadotropin secretion in the pituitary [36]. On the other hand, it has been suggested that a G1-mediated activation of protein phosphatase...
is responsible for the direct antiproliferative effect of GnRH in human cancer cells. Depending on the cancer cell type, this coupling may result in the production of different receptor conformation and signaling complexes that could explain why GnRH ligands do not exert a significant adverse effect on the pituitary cells [11].

**GnRH receptor-mediated growth inhibition**

The competitive binding study was performed to evaluate the role of GnRH-R in the antiproliferative activity of GnRH analogs in DU145, LNCaP, and OVCAR-3 cell lines. The effect of all compounds with significant antiproliferative activity compared to their parent peptide was abolished after 2 h pretreatment with the 100 μM triptorelin. Triptorelin pretreatment affected the activity of antiproliferative activity of all compounds (1–6) in DU145, LNCaP, and OVCAR-3 cell lines (Fig. 3A–C). The diminished antitumor activity of peptide analogs after pretreatment of GnRH receptor-positive cells with a superagonist, triptorelin, is suggestive of selective receptor-mediated action of the GnRH analogs in these cells. Inhibition of the mitogenic signal transduction pathways of the epidermal growth factor receptor in prostate, endometrial, ovarian, and breast cancer cell lines has been reported to be responsible for the receptor-mediated antiproliferative activity of GnRH analogs [37]. This suggests that the antiproliferative effect of GnRH analogs is mediated via overexpressed GnRH receptors in LNCaP, OVCAR-3, and DU-145 cells, which could explain why no significant activity was observed in GnRH receptor low-expressing cells (SKOV-3).

**The impact of sex steroid hormone on the antitumor activity**

Previous studies have shown the regulatory function of sex steroids on GnRH-R expression and the antiproliferative activity of GnRH agonists [38, 39]. Therefore, we studied the relationship between the growth inhibitory effect of GnRH analogs and steroid hormones in a steroid depleted media (CSS). Prostate and ovarian cancer cells (DU145, LNCaP, and OVCAR-3) were treated with GnRH analogs with or without DHT and E2, respectively. Using CSS
media that contained serum depleted of low molecular-weight lipophilic compounds such as steroid hormones resulted in a significant reduction \( (p < 0.05) \) in the sensitivity of DU145 and LNCaP cells to compound 1–4 and control peptides at 50 nM was added. Following 48 h incubation, MTT assay was performed (mean \( \pm \) SEM, assay performed in three independent experiments, each in triplicate). A one-way ANOVA was used \( (*p < 0.05, **p < 0.01, ***p < 0.001) \), comparison was made between pretreated and untreated groups. DMSO at 0.5% was used as a negative control for its potential impact on the cell proliferation

Fig. 3 Receptor-mediated antiproliferation in (A) DU145, (B) LNCaP, and (C) OVCAR-3 cells. Triptorelin ([w6]GnRH) at 100 µM was used for pretreatment and saturation of GnRH receptors in the cells. The media was removed, and fresh media together with compounds 1–6 and control peptides at 50 mM was added. Following 48 h incubation, MTT assay was performed (mean \( \pm \) SEM, assay performed in three independent experiments, each in triplicate). A one-way ANOVA was used \( (*p < 0.05, **p < 0.01, ***p < 0.001) \), comparison was made between pretreated and untreated groups. DMSO at 0.5% was used as a negative control for its potential impact on the cell proliferation

Fig. 4 Effect of the reconstitution of the cell growth media with steroid hormone (DHT and E2) on the sensitivity of prostate cancer cells (A) DU145, (B) LNCaP, and (C) ovarian cancer cells (OVCAR-3) to GnRH analogs. Cells were grown in steroid-free (CSS) media for 48 h. One group of cells was reconstituted with E2 (5 nM) or DHT (50 nM). Another group was treated with lipopeptides at 50 µM. Reconstituted cells were treated with compounds 1–6 and control peptides for a further 48 h. \( *p < 0.05, **p < 0.01, ***p < 0.001 \) for experiments performed in normal media vs. CSS media. \( *p < 0.05, **p < 0.01, ***p < 0.001 \) for experiments performed in steroid reconstituted media in comparison with CSS and normal media

media that contained serum depleted of low molecular-weight lipophilic compounds such as steroid hormones resulted in a significant reduction \( (p < 0.05) \) in the sensitivity of DU145 and LNCaP cells to compound 1–4 and 6, and [w6]GnRH (Figs 4A, B). However, after the addition of DHT to the media, the antiproliferative activity was returned. Our findings were in line with previous reports where up to 119% upregulation of GnRH receptor expression in GnRH receptor-positive prostate cancer cells was observed that was dependent on the presence of DHT. This steroid-dependent upregulation of the GnRH receptors resulted in the hypersensitivity of prostate cancer cells and significant antiproliferative activity of GnRH agonists [38]. In another report, receptor upregulation was similarly observed when cells were treated with a GnRH agonist and DHT [40]. The higher activity of the lipopeptides and
triptorelin in DU145 might be explained by an upregulation of GnRH receptors in the presence of DHT.

In OVCAR-3 cells, the presence and absence of steroid E2 conversely affected the sensitivity of these cells compared to the prostate cancer cells. In CSS media, the sensitivity of OVCAR-3 cells was increased to the growth inhibitory effect of GnRH analogs, while the addition of E2 to CSS media significantly decreased the antiproliferative activity of GnRH analogs (Fig. 4C). These results were consistent with previous studies that reported estrogen not only has a mitogen effect on OVCAR-3 but also downregulated the GnRH receptor expression at mRNA level [39].

**Gonadotropin release assay**

The effect of the GnRH analogs on the gonadotropin (LH and FSH) release was examined in vitro by incubating rat pituitary cells with the GnRH analogs and control peptides (GnRH and [w6]GnRH) for 2 h. All [w6]GnRH-based compounds (2, 3, and 6) significantly stimulated the FSH release from dispersed pituitary cells compared to the negative control at 0.5 and 5 nM. The highest increase in the FSH release was caused by compounds 3 and 6 up to 1.3 and 1.4 ng/ml, respectively, which was comparable with the effect of the control superagonist, triptorelin, and was significantly higher than PBS negative control (0.9 ng/ml, p > 0.05, Fig. 5A).

Same compounds (2, 3, and 6) significantly stimulated the level of LH release at 0.5 and 5 nM, compared to PBS control (p > 0.05, Fig. 5B). The increase in the level of LH by the lipid-modified peptide derivatives was higher than that of triptorelin, suggesting a more efficient activation of the GnRH receptors upon binding. Although compound 1 from the GnRH-based group caused a significant increase in LH release at a higher concentration (5 nM), this effect was lower than that of [w6]GnRH-based peptide derivatives. The higher LH release stimulatory effect of [w6]GnRH-based lipopeptides (2, 3, and 6) could be due to their higher stability, allowing longer interaction with the receptor in addition to leading to a higher direct growth inhibitory activity. These results indicate that while lipid modification has increased the metabolic stability in the peptide, it has not adversely affected the gonadotropin release from the pituitary cells, which in turn suggests an efficient binding to GnRH receptors has been preserved.

**Experimental section**

**General**

Normal Fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640, HBSS, HEPES, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), and all solvents used for assays were sourced from Sigma-Aldrich (Castle Hill, NSW, Australia). Tissue culture flasks (TPP 75 cm²) and normal 96-well plates were ordered from Becton Dickinson; High-affinity microplates (9018) were purchased from Corning (USA). Charcoal Stripped FBS was sourced from Life Technologies (Gibco). LH and FSH enzyme-linked immunosorbent assay (ELISA) kits were purchased from Uscn Life Science Inc., Wuhan, China. Lipid-conjugated analogs had been previously synthesized by Dr. Mansfeld and Dr. Varamini, were used [27].

**Peptides preparation for cell assays**

The GnRH peptide and its analogs were synthesized according to a previously published method [27]. The stock solution was prepared at 1000 µM concentration using DMSO and buffered water. The stock solutions were diluted with 5% DMSO and buffered H₂O to form 500 µM, 100 µM, 1 µM solutions. GnRH and [w6]GnRH were used as controls to compare with the modified GnRH compounds. 10% DMSO
was used as a negative control and SDS as a positive control. The sequence of different peptides is shown in Table 1.

**Cell lines and culture media**

Different cell lines were used with various levels of sensitivity in response to steroids. The LNCaP (GnRH receptor-positive; androgen-sensitive prostate adenocarcinoma), PC3 and DU145 (GnRH receptor-positive; androgen-independent human carcinoma), OVCAR-3 (GnRH receptor-positive; steroid hormone-sensitive ovarian carcinoma), and SKOV-3 (GnRH receptor low-expressing; estrogen-resistant ovarian carcinoma) human cell lines were used. Cells were provided by Professor Judith Clements at the Translational Research Institute (TRI), Queensland University of Technology, and Professor Rodney Minchin, School of Biomedical Sciences, The University of Queensland. Cells were grown in RPMI 1640 medium with 10% FBS at 37 °C in an incubator in a humidified atmosphere of 5% CO₂; 95% air. For steroid assay, 10% charcoal-stripped serum was used in RPMI 1640 media.

**In vitro antiproliferative activity studies**

Cells were grown in the incubator for few days, and media was changed on a regular basis. The cells with 70% over confluence were passaged. For in vitro studies, 90 µl of cells were seeded onto each well in the 96-well flat-bottom plates with a concentration of 20,000 cells/well approximately. GnRH analogs were prepared at 1, 10, 25, 50, and 100 µM concentrations. The growth medium, 10% DMSO, and Sodium Dodecyl Sulfate (SDS) solution were used as assay controls. Concentrations of natural GnRH and [w⁶]GnRH (triptorelin) were also prepared as positive controls. After seeding the cells and overnight incubation, 10 µl of each compound was added in triplicates, and plates were incubated at 37 °C in the humidified atmosphere for 48 h. Plates were taken out from the incubator after 48 h to add 10 µl of MTT in 570 nm, and data was recorded. The assay was performed using the GnRH-receptor positive cell lines, DU145, LNCap, and OVCAR-3. When grown to 70% confluence, cells were seeded onto 96-well flat-bottom plates and pretreated with 100 µM [w⁶]GnRH for 2 h. GnRH compounds (50 µM) were added in triplicates, and MTT assay was performed after 48 h of incubation with compounds (refer to section 2.4).

**Competitive study for receptor-mediated antiproliferation**

For receptor-mediated antiproliferation assay, a trial experiment was performed using different [w⁶]GnRH concentrations (50, 100, and 500 µM) and pretreatment with cells at different incubation times (2, 4, 8, and 24 h). A 2 h-incubation time with 100 µM [w⁶]GnRH was used due to better results achieved for this concentration at this duration. The assay was performed using the GnRH-receptor positive cell lines, DU145, LNCap, and OVCAR-3. When grown to 70% confluence, cells were seeded onto 96-well flat-bottom plates and pretreated with 100 µM [w⁶]GnRH for 2 h. GnRH compounds (50 µM) were added in triplicates, and MTT assay was performed after 48 h of incubation with compounds (refer to section 2.4).
Steroid treatment studies

Lipidated analogs were used, and 10% FBS media was replaced with 10% charcoal-stripped serum media into two flasks of cells. After 48 h, one flask was taken, and cells were seeded onto plates following the same protocol as in vivo study (refer to section 2.4). In the second flask, only media was changed, 50 nM DHT was added to DU145 and LNCap cells, and 5 nM 17β-estradiol for OVCAR-3 followed by another 48 h incubation. MTT was performed on the incubated plates after 48 h (refer to section 2.5). The data was recorded for both the plates and compared with normal media assay.

In the steroid treatment experiment, cells with 70% confluence were washed twice with PBS. Then the 10% charcoal-stripped CSS (CSS) media was added to the flask. Cells were divided into two parts; one part was seeded in 96-well plates after 48 h incubation and treated with compounds 1–6 and control peptides at 50 µM to perform the MTT assay. Another part was treated with fresh CSS media containing 5 nM 17β-estradiol (E2) or 50 nM dihydrotestosterone (DHT) for an additional 48 h incubation. Treated cells were plated in 96-well plates. Compounds were added at 50 µM followed by MTT assay after 48 h incubation.

Gonadotropin release assay

Pituitary cells were isolated from rat pituitary following previously described methods (refer to section 2.5). Rat pituitary cells were plated in flat-bottom 96-well plates at a density of 3 × 10^5 cells/well and incubated at 37 °C for 72 h. Plates were centrifuged at 1200 g for 10 min. Cell media was replaced by challenge media containing DMEM with 0.1% BSA. Then cells were incubated at 37 °C for 2 h with the GnRH analogs 1–6 and control peptides (GnRH and triptorelin) at the different concentrations (0.5, 5, and 50 nM) at 10 µl. The level of LH and FSH gonadotropins was quantified using a commercial ELISA kit according to the manufacturer’s instructions.

Conclusion

Several conditions are currently treated by GnRH agonists as powerful therapeutic agents in sex hormone-related tumors (e.g., prostate, ovarian, endometrial, and some types of breast cancers). A GnRH receptor-mediated direct antiproliferative activity was observed following lipid-modification, together with D-amino acid substitution. Between two groups of peptide derivatives that we synthesized, [w6] GnRH-based lipopeptides were shown to be more promising for the future development of therapeutic candidates effective in ovarian and prostate cancers. In particular, lipopeptides 3 that showed the highest in vitro metabolic stability, was the most potent analog in inhibiting cancer cell growth, superior to triptorelin. This analog showed comparable FSH release activity with triptorelin and higher LH release efficacy in pituitary cells than this superagonist. These findings make compound 3 a promising candidate for the development of new GnRH agonists as well as new targeted moieties to be conjugated to different drug delivery systems to treat hormone-sensitive and -refractory prostate and ovarian cancers.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval This research was conducted in accordance with NHMRC Australia’s Code of Practice, approved by and performed in compliance with the guidelines of The University of Queensland Ethics Committee (ethics approval number: 2009000661).

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References

1. Marques P, Skorupskaite K, George JT, Anderson RA. Physiology of GnRH and Gonadotropin Secretion. [Updated 2018 Jun19]. In: Feingold KR, Anawalt B, Boyce A, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000. Available from: https://www.ncbi.nlm.nih.gov/books/NBK279070/.
2. Mahesh VB, Dhindsa DS, Anderson E, Kalra SP. Regulation of ovarian and testicular function. US: Springer; 2012.
3. Vickery BH, Nestor J, Hafez ES. LH and its analogs: contraceptive and therapeutic applications. Netherlands: Springer; 2012.
4. Höflken K. LH-RH agonists in oncology. Berlin Heidelberg: Springer; 1988.
5. Fraser HM. LHRH analogues: their clinical physiology and delivery systems. Baillière’s Clin Obstet and Gynaecol. 1988;2:639-658.
6. Schally AV, Engel JB, Pinski J, Block NL. LHRH Analogs. 2013:331–40. https://doi.org/10.1016/j.bmrc.2013.01.003.

7. Marelli MM, Moretti RM, Mai S, Procacci P, Limonta P. Gonadotropin-releasing hormone agonists reduce the migratory and the invasive behavior of androgen-independent prostate cancer cells by interfering with the activity of IGF-I. Int J Oncol. 2007;30:261–71.

8. Eidne KA, Flanagan CA, Harris NS, Millar RP. Gonadotropin-Releasing Hormone (Gnrh)-Binding Sites in Human-Breast Cancer Cell Lines and Inhibitory Effects of Gnrh Antagonists. J Clin Endocrinol Metab. 1987;64:425–32.

9. Lin QY, Wang YF, Weng HN, Sheng XJ, Jiang QP, Yang ZY. Influence of gonadotropin-releasing hormone agonist on the effect of chemotherapy upon ovarian cancer and the prevention of chemotherapy-induced ovarian damage: an experimental study with nu/nu athymic mice. J Zhejiang Univ Sci B. 2012;13:894–903. https://doi.org/10.1631/jzus.B1100369.

10. Kakar SS, Jennes L. Expression of gonadotropin-releasing hormone and gonadotropin-releasing hormone receptor mRNAs in various non-reproductive human tissues. Cancer Lett. 1995;98:57–62. https://doi.org/10.1016/S0304-3835(06)80108-8.

11. Gründker C, Emons G. The role of gonadotropin-releasing hormone in cancer cell proliferation and metastasis. Front in Endocrinol. 2017:8. https://doi.org/10.3389/fendo.2017.00187.

12. Gründker C, Emons G. Role of gonadotropin-releasing hormone (Gnrh) in ovarian cancer. Cells. 2021;10. https://doi.org/10.3390/cells10020437.

13. Saad F, Shore N, Zhang T, Sharma S, Cho HK, Jacobs IA. Emerging therapeutic targets for patients with advanced prostate cancer. Cancer Treat Rev. 2019;76:1–9. https://doi.org/10.1016/j.ctrv.2019.03.002.

14. Kim TJ, Koo KC. Current status and future perspectives of checkpoint inhibitor immunotherapy for prostate cancer: a comprehensive review. Int J Mol Sci. 2020;21. https://doi.org/10.3390/ijms21115484.

15. Fontana F, Marzagalli M, Montagnani M, Raimondi M, Moretti RM, Limonta P. Gonadotropin-releasing hormone receptors in prostate cancer: molecular aspects and biological functions. Int J Mol Sci. 2020;21. https://doi.org/10.3390/ijms21249511.

16. Parborell F, Irusta G, Celin AR, Tesone M. Regulation of ovarian angiogenesis and apoptosis by GnRH-I analogs. Mol Reprod Dev. 2008;75:623–31. https://doi.org/10.1002/mrd.20806.

17. Van Poppel H, Abrahamsson PA. Considerations for the use of gonadotropin-releasing hormone agonists and antagonists in patients with prostate cancer. Int J Urol. 2020;27:830–7. https://doi.org/10.1111/iju.14303.

18. Morgan K, Stewart AJ, Miller N, Mullen P, Muir M, Dodds M, et al. Gonadotropin-releasing hormone receptor levels and cell context affect tumor cell responses to agonist in vitro and in vivo. Cancer Res. 2008;68:6331–40. https://doi.org/10.1158/0008-5472.Can-08-0197.

19. Schally AV, Nagy A. Cancer chemotherapy based on targeting of cytokotoxic peptide conjugates to their receptors on Ers. J Endocrinol. 1999;141:1–14. https://doi.org/10.1530/eje.0.1410001.

20. Li X, Taratula O, Taratula O, Schumann C, Minko T. LH-RH-targeted drug delivery systems for cancer therapy. Mini Rev Med Chem. 2017;17:258–67. https://doi.org/10.2174/138955751666161013111155.

21. Emmons G, Gorchev G, Harter P, Wimmerperger P, Stähle A, Hanker L, et al. Efficacy and safety of AEZS-108 (LHRH agonist linked to doxorubicin) in women with advanced or recurrent endometrial cancer expressing LHRH receptors: a multicenter phase 2 trial (AGO-1014). Int J Gynecol Cancer. 2014;24:260–5. https://doi.org/10.1097/IGC.0000000000000444.

22. Miller DS, Scambia G, Bondarenko I, Westermann AM, Oktvin A, Oza AM, et al. ZoptEC: Phase III randomized controlled study comparing zoptarelin with doxorubicin as second line therapy for locally advanced, recurrent, or metastatic endometrial cancer (NCT01767155). J Clin Oncol 2018;36:5503 https://doi.org/10.1200/JCO.2018.36.15_suppl.5503.

23. Curtis KK, Sarantopoulos J, Northfelt DW, Weiss GJ, Barnhart KM, Whisnant JK, et al. Novel LHRRH-receptor-targeted cytotoxic peptide, EP-100: first-in-human phase I study in patients with advanced LHRRH-receptor-expressing solid tumors. Cancer Chemother Pharm. 2014;73:931–41. https://doi.org/10.1007/s00280-014-2424-x.

24. Toth I, Flinn N, Hillery A, Gibbons WA, Artursson P. Lipidic conjugates of luteinizing-hormone-releasing hormone (Lhrh)+ and thyrotropin-releasing hormone (Trh)+ that release and protect the native hormones in homogenates of human intestinal epithelial (Caco-2) cells. Int J Pharmaceutics. 1994;105:241–7.

25. Rink R, Arkema-Meter A, Baudoin I, Post E, Kuijpers A, Nellmans SA, et al. To protect peptide pharmaceuticals against peptides. J Pharmacol Toxicol Methods. 2010;61:210–8. https://doi.org/10.1016/j.vascn.2010.02.010.

26. Toth I. A novel chemical approach to drug-delivery—lipidic amino-acid conjugates. J Drug Target. 1994;2:217–39.

27. Mansfeld FM, Toth I. Lipidated analogues of luteinizing hormone-releasing hormone (LHRH) reduce serum levels of follicle-stimulating hormone (FSH) after oral administration. Int J Pharm. 2012;439:216–22. https://doi.org/10.1016/j.ijpharm.2012.09.038.

28. Moradi SV, Mansfeld FM, Toth I. Synthesis and in vitro evaluation of glycosyl derivatives of luteinizing hormone-releasing hormone (LHRH). Bioorg Medicinal Chem. 2013;21:4259–65. https://doi.org/10.1016/j.bmc.2013.04.068.

29. Kluth L, Shariat S, Kratzik C, Tagawa S, Sonpavde G, Rieken M, et al. The hypothalamic–pituitary–gonadal axis and prostate cancer: implications for androgen deprivation therapy. World J Urol. 2014;32:669–76. https://doi.org/10.1007/s00345-013-1157-5.

30. Hollosy F, Lorand T, Orfi L, Eros D, Keri G, Idei M. Relationship between lipophilicity and antitumor activity of molecule library of Mannich ketones determined by high-performance liquid chromatography, clogP calculation and cytotoxicity test. J Chromatogr B. 2002;768:361–8. https://doi.org/10.1016/S1570-0232(02)00004-1. P0S1570-0232(01)00004-1.

31. Skinner DC, Albertson AJ, Navratil A, Smith A, Mignot M, Talbott H, et al. Effects of gonadotrophin-releasing hormone outside the hypothalamic-pituitary-reproductive axis. J Neuroendocrinol. 2009;21:282–92. https://doi.org/10.1111/j.1365-2826.2009.01842.x.

32. Mezo G, Manea M. Receptor-mediated tumor targeting based on peptide hormones. Expert Opin Drug Deliv. 2010;7:79–96. https://doi.org/10.1517/17425240903418410.

33. Chen CL, Cheung LW, Lau MT, Choi JH, Auersperg N, Wang HS, et al. Differential role of gonadotropin-releasing hormone on human ovarian epithelial cancer cell invasion. Endocrine 2007;31:311–20.

34. Pfleger KD, Bogerd J, Millar RP. Conformational constraint of mammalian, chicken, and salmon GnRhS, but not GnRH II, enhances binding at mammalian and nonmammalian receptors: evidence for preconfiguration of GnRH. Mol Endocrinol. 2002;16:2155–62.

35. Dharap SS, Wang Y, Chandna P, Khandare JJ, Qiu B, Gunaseelan M, et al. Tumor-specific targeting of an anticancer drug delivery system by LHRRH peptide. P Natl Acad Sci USA. 2005;102:12962–7. https://doi.org/10.1073/pnas.0504274102.

36. Everest HM, Hislop JN, Harding T, Uney JB, Flynn A, Millar RP, et al. Signal and Antiproliferative Effects Mediated by GnRH Receptors After Expression in Breast Cancer Cells Using Recombinant Adenovirus. Endocrinology. 2001;142:4663–72. https://doi.org/10.1210/endo.142.11.8503.

37. Grundker C, Volker P, Schulz KD, Emons G. Luteinizing hormone-releasing hormone agonist triptorelin and antagonist...
38. Leuschner C, Enright FM, Gawronska-Kozak B, Hansel W. Human prostate cancer cells and xenografts are targeted and destroyed through luteinizing hormone releasing hormone receptors. Prostate. 2003;56:239–49. https://doi.org/10.1002/pros.10259

39. Kang SK, Choi K-C, Tai C-J, Auersperg N, Leung PCK. Estradiol regulates gonadotropin-releasing hormone (GnRH) and its receptor gene expression and antagonizes the growth inhibitory effects of gnrh in human ovarian surface epithelial and ovarian cancer cells. Endocrinology. 2001;142:580–8. https://doi.org/10.1210/endo.142.2.7982

40. Angelucci C, Lama G, Iacopino F, Ferracuti S, Bono AV, Millar RP, et al. GnRH receptor expression in human prostate cancer cells is affected by hormones and growth factors. Endocrine. 2009;36:87–97. https://doi.org/10.1007/S12020-009-9195-X

41. Varamini P, Mansfeld FM, Giddam AK, Steyn F, Toth I. New gonadotropin-releasing hormone glycolipids with direct anti-proliferative activity and gonadotropin-releasing potency. Int J Pharm. 2017;521:327–36. https://doi.org/10.1016/j.ijpharm.2017.02.054

42. Moradi SV, Varamini P, Toth I. Evaluation of the biological properties and the enzymatic stability of glycosylated luteinizing hormone-releasing hormone analogs. AAPS J. 2015;17:1135–43. https://doi.org/10.1208/s12248-015-9769-x