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 any significant direct antitumor activity to make them suitable for hormone-refractory reproductive cancers. In this study, we modified GnRH peptide and triptorelin to improve their stability, pharmacokinetic properties, and potency and subsequently broaden their clinical applications in cancer. We investigated biological properties of lipid-modified GnRH analogs, with/without D-amino acid substitution at position 6 to yield GnRH- and triptorelin-based derivatives, respectively, in prostate and ovarian cancer cells. We showed that the improved 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) and gonadotropin-releasing potency. Furthermore, sex steroids played significant but contrasting roles in regulating the direct antiproliferative activity of the lipopeptides in cancer cells. The superior activity of these GnRH analogs over triptorelin 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.

1. 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 decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) controlling the reproductive axis and produced by neurons located in cell bodies of the hypothalamic-preoptic region [1, 2]. Following transportation 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 hypophyseal-gonadal axis via down-regulation, 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 down-regulation 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]. A strong antiproliferative, antimetastatic, and antiangiogenic activity has been observed after activating the GnRH receptors in tumor cells by its agonists [11]. These effects have been shown to be directly dependent on the level of GnRH receptor expression in particular tumor cells [12]. 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 [13]. Several GnRH receptor-targeted delivery systems have been developed using different derivatives of GnRH peptide [14] and a few reached clinical trials [15–17]. 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 [18]. 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 [19]. 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 [20].

We previously showed that lipid-modified GnRH analogs showed significantly improved metabolic stability and membrane permeability in different *in vitro* models [21]. 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 [21]. 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 Leu^7 (compounds **3** and **5**) or to the C-terminus (compound **6**). D-Tryptophan ($D\text{-}Trp^6$ or w^6) substitution was performed to produce [w^6]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 [22].

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.

2. 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 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 [23]. 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 [24]. 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.

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

Peptide name	**Amino acid sequence**	**$t_{1/2}$ [min] [21]**
1 C12[Q^1]GnRH	C12[a] QHWSYGLRPG	48.5 ± 7.1
2 C12[Q^1][w^6]GnRH	C12[a] QHWSYwLRPG	225.4 ± 10.4
3 [w^6][$C12^7$]GnRH	pEHWSYwC12[a]RPG	167.8 ± 12.5
4 [$C12^6$]GnRH	pEHWSYC12[a]LRPG	79.5 ± 8.7
5 [$C12^7$]GnRH	pEHWSYGC12[a]RPG	82.6 ± 9.6
6 [w^6]GnRH-C12	pEHWSYwLRPGC12[a]	146.9 ± 10.1
GnRH	pEHWSYGLRPG	6.4 ± 2.3
[w^6]GnRH (triptorelin)	pEHWSYwLRPG	110.1 ± 15.9
[a] C12-lipoamino acid		

2.1. 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 [25], the dose by which they exert these activities varies GnRH receptor-positive peripheral tissues [26]. At low (nanomolar) concentrations, GnRH ligands increase cell proliferation while they inhibit cell proliferation at high (micromolar) concentrations [27]. This phenomenon has been shown to be due to the differential expression profiles of GnRH receptors [26]. 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 IC_{50} (μM) values (Table 2). Triptorelin (shown as [w^6]GnRH) was used as the parent peptide (control) for [w^6]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% (IC_{50} = 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 (IC_{50} > 100, Table 2). In the [w^6]GnRH-based group, compounds **3** and **6** with IC_{50} values of 29 and 33 μM, respectively, showed the highest growth inhibition in DU145 compared to their control peptide [w^6]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 [w^6]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 IC_{50} values lower than their parent peptides. Compound **3** with an IC_{50} value of 16 μM showed the highest growth inhibition in OVCAR-3 compared to its parent peptide [w^6]GnRH and other GnRH analogs (Table 2). While compounds **4** and **5** in the GnRH-based group exhibited a significant growth inhibitory effect compared to GnRH peptide ($p < 0.05$), their direct antiproliferative activity was not significantly higher than triptorelin.

The GnRH receptor-low expressing hormone-resistant ovarian cancer cell line (SKOV-3) showed to be resistant to the antiproliferative activity of all GnRH analogs and natural GnRH peptides with all IC_{50} values obtained above 100 μM (Table 2).

The results from antiproliferative studies indicated that N-terminal modification of GnRH with the lipid, regardless of D-Trp modification in the middle of the sequence, does not improve the direct antitumor activity. This result was not in line with the metabolic stability of these peptide analogs in Caco-2 cell homogenates, where analog **2** showed the highest stability among all other peptides. These findings suggest that while N-terminal modification of GnRH peptide by lipid together with mid-sequence substitution with D-Trp could increase the metabolic stability above two-fold, it did not improve the direct antiproliferative activity. In contrast, D-Trp modification together with either mid-sequence or C-terminal lipid-conjugation resulted in significantly improved direct antiproliferative activity in all GnRH-overexpressing cell lines. This could be due to the enhanced conformational stability of the peptide and the formation of a β-II turn for greater receptor binding. This, in turn, improved GnRH receptor-mediated antiproliferative activity [28]. Plausibly, the position of lipoamino acid residues on the C-terminus and middle of the sequence of the GnRH backbone contributed to the enhanced antitumor and gonadotropin release activity of GnRH lipopeptides. Considering the increase in metabolic stability and direct antiproliferative activity in the peptide analogs, compound **3** was found to be the most promising derivative for future investigations (see Fig. 1 for the chemical structure).

Table 2
Antiproliferative Activity of GnRH Analogues in Different Cell Line

Lipo-GnRH Analogues	**1**	**2**	**3**	**4**	**5**	**6**	**GnRH**	**[w^6]GnRH (triptorelin)**
DU145 IC_{50} (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 IC_{50} (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 IC_{50} (μM)	89.4 ± 3.3	90.1 ± 5.1	16.0 ± 3.3*	51.3 ± 4.3*	58.6 ± 3.9*	30.3 ± 2.8*	> 100	67.7 ± 3.1
SKOV-3 IC_{50} (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).

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

GnRH analogs were also tested on the proliferation of non-cancerous 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 [29]. 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* $G_{q/11}$ 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 [30]. On the other hand, it has been suggested that a G_i-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 [31].

2.3. 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 [32]. 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).

2.4. 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 [33, 34]. 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 **6**, and [w^6]GnRH (Fig. 4A and 4B). 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 [35]. In another report, receptor upregulation was similarly observed when cells were treated with a GnRH agonist and DHT [36]. 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 [34].

2.5. 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 [w^6]GnRH) for 2 h. All [w^6]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 [w^6]GnRH-based peptide derivatives. The higher LH release stimulatory effect of [w^6]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.

3. Experimental Section

3.1. 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 (TPP1 75 cm^2) 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 [37].

3.2. Peptides preparation for cell assays

The GnRH peptide and its analogs were synthesized according to a previously published method [37]. 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 [w^6]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.

3.3. 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^o C in an incubator in a humidified atmosphere of 5% CO_2; 95% air. For steroid assay, 10% charcoal-stripped serum was used in RPMI 1640 media.

3.4. 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 approx. 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 [w^6]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^{o}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 each well and incubated for 4 h. Then 100 μl of acidified Isopropanol (0.1N HCl) was added to each well to dissolve

formazan crystals. Sonicator was used to mix till the media turns purple, followed by pipetting each well. The absorbance was measured using Spectramax 250 microplate reader at 570 nm, and data was recorded. All the cell proliferation assays were repeated three independent times in triplicate.

3.5. *Cytotoxicity study against non-cancerous Peripheral Blood Mononuclear Cells (PBMCs) and rat pituitary cells*

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). PBMCs were isolated from human blood and cultured according to a previously published method [38]. Briefly, 4 ml of blood taken from a healthy volunteer adult were centrifuged at 400 g for 30 min on Ficoll.

From the plasma–Ficoll interface, the mononuclear cell layer was collected, followed by three times washing with RPMI 1640.
Cells were seeded at 1×10^6 cells/ml (80 µl) in a 96-well flat-bottom plate. Phytohemagglutinin (10 µg/ml, 10 µl) was used to activate the PBMCs during the incubation time. After 1 h incubation at 37°C, compounds were added (50 and 100 µM, 10 µl) in 10% DMSO/PBS (10 µl/well). An MTT assay was performed after 48 h incubation (refer to section 2.4).

Pituitaries were received from Australian Institute for Bioengineering and Nanotechnology Research Animal Facility (AIBN) at The University of Queensland having been dissected from rats (male, Sprague–Dawley, 6–8 weeks, 120–180 g). Pituitary cells were cultured according to a previously published method [39]. Briefly, the anterior pituitaries removed immediately after euthanasia were rinsed with HBSS-HEPES (25 mM, pH 7.2), and then chopped into small pieces using a razor blade. Pituitary fragments were incubated in a collagenase enzyme solution (1 mg/ml dissolved in 1% bovine serum albumin) for 1 h at 37°C. Using a cell strainer (Costar), cell clumps were removed. The suspension was centrifuged at 400 *g* for 10 min. After the supernatant was decanted, cells were resuspended in 10% FBS in DMEM media. Then cells were counted and 90 µl/well plated at 4×10^5 cells/ml. After 1 h incubation, compounds dissolved in 10% DMSO/PBS (50 and 100 µM) were added to each well (10 µl), and MTT assay was performed after 48h incubation (refer to section 2.4).

3.6. Competitive study for receptor-mediated antiproliferation

For receptor-mediated antiproliferation assay, a trial experiment was performed using different [w^6]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 [w^6]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 [w^6]GnRH for 2 hours. GnRH compounds (50 µM) were added in

triplicates, and MTT assay was performed after 48 hours of incubation with compounds (refer to section 2.4).

3.7. 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 vitro* 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 17ß-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 FBS (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 17ß-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.

3.8. 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.

4. 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, [w^6]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.

Declarations

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Consent to participate (include appropriate statements): N/A

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Figures

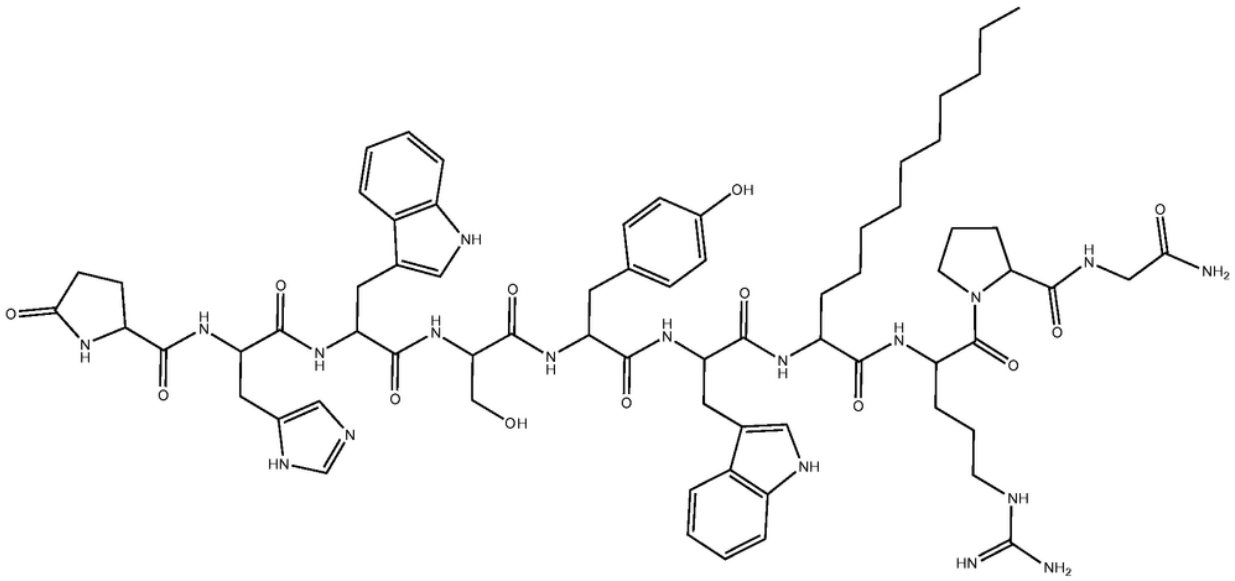

Figure 1

Chemical structure of compound 3 ([w6][C127]GnRH)

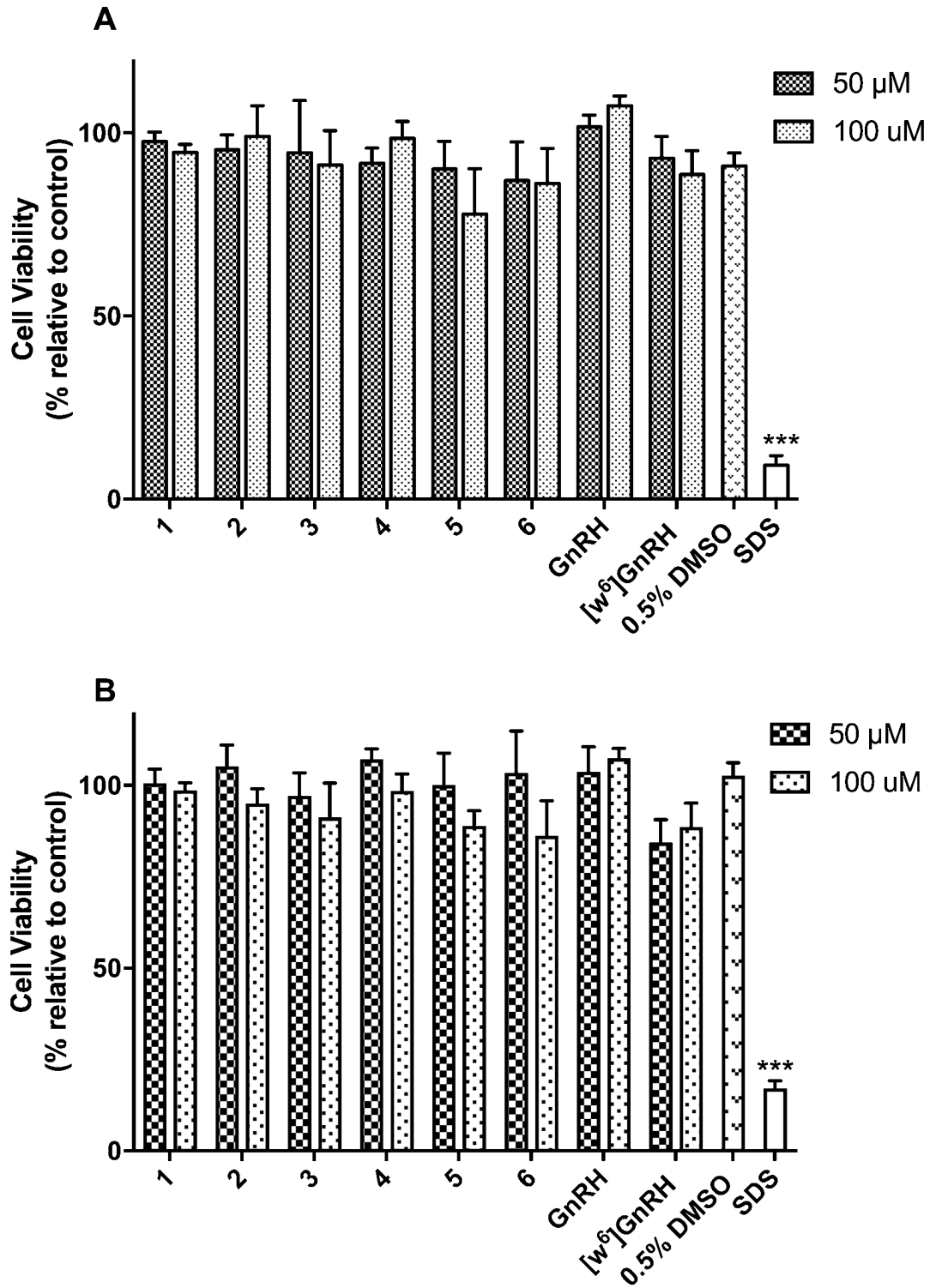

Figure 2

Toxicity study of GnRH agonist and peptide control (GnRH and [w6]GnRH) against A) PBMC and B) Pituitary cells. Data are presented as a percentage of cells treated with negative control, PBS, (mean ± SD, performed in three independent experiments, each in triplicate) following 48 h incubation. A one-way ANOVA followed by Dunnett's post-hoc test was used, and comparison was made with the 0.5% DMSO control group (*** $p < 0.001$)

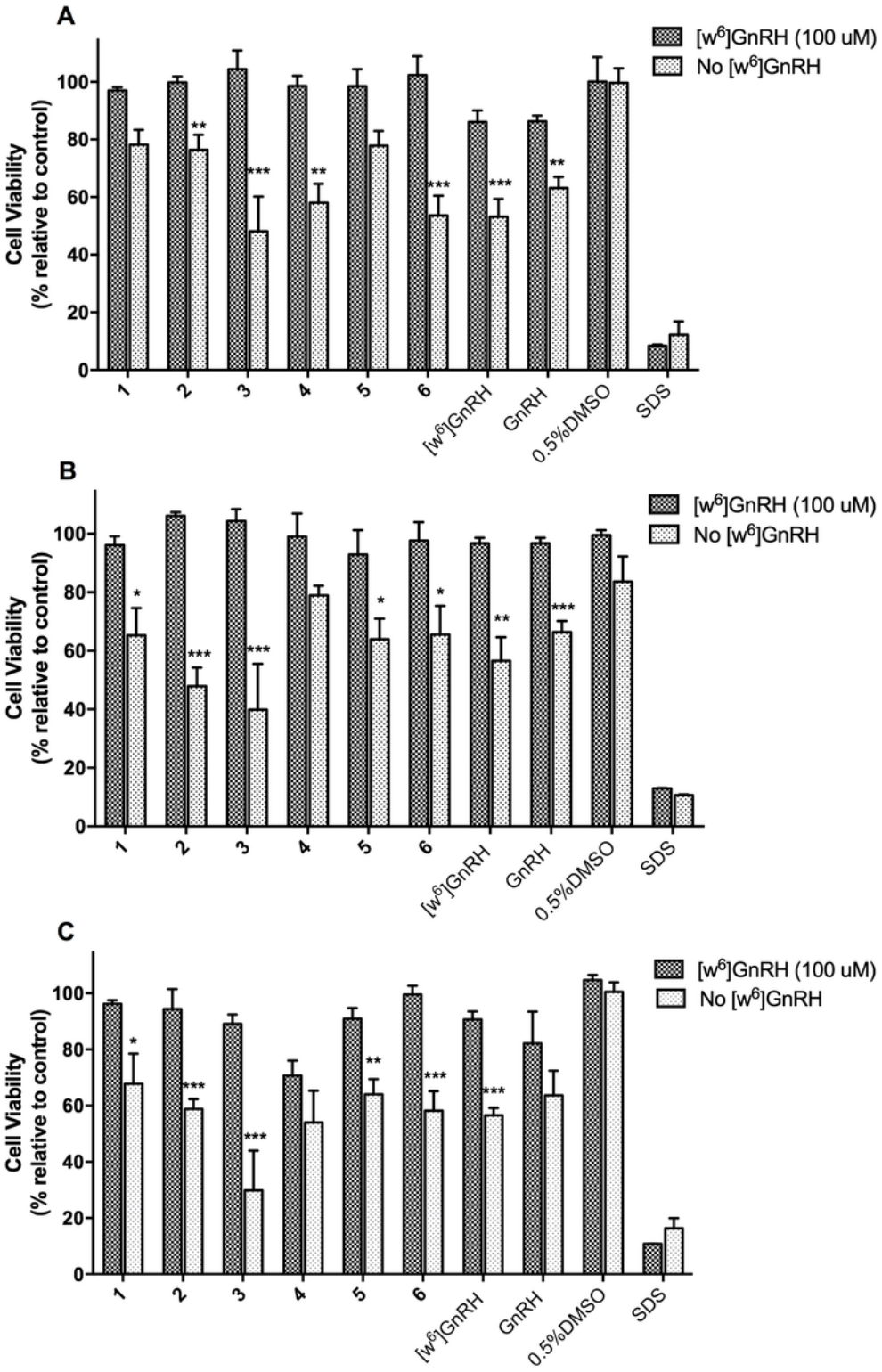

Figure 3

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 ± 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

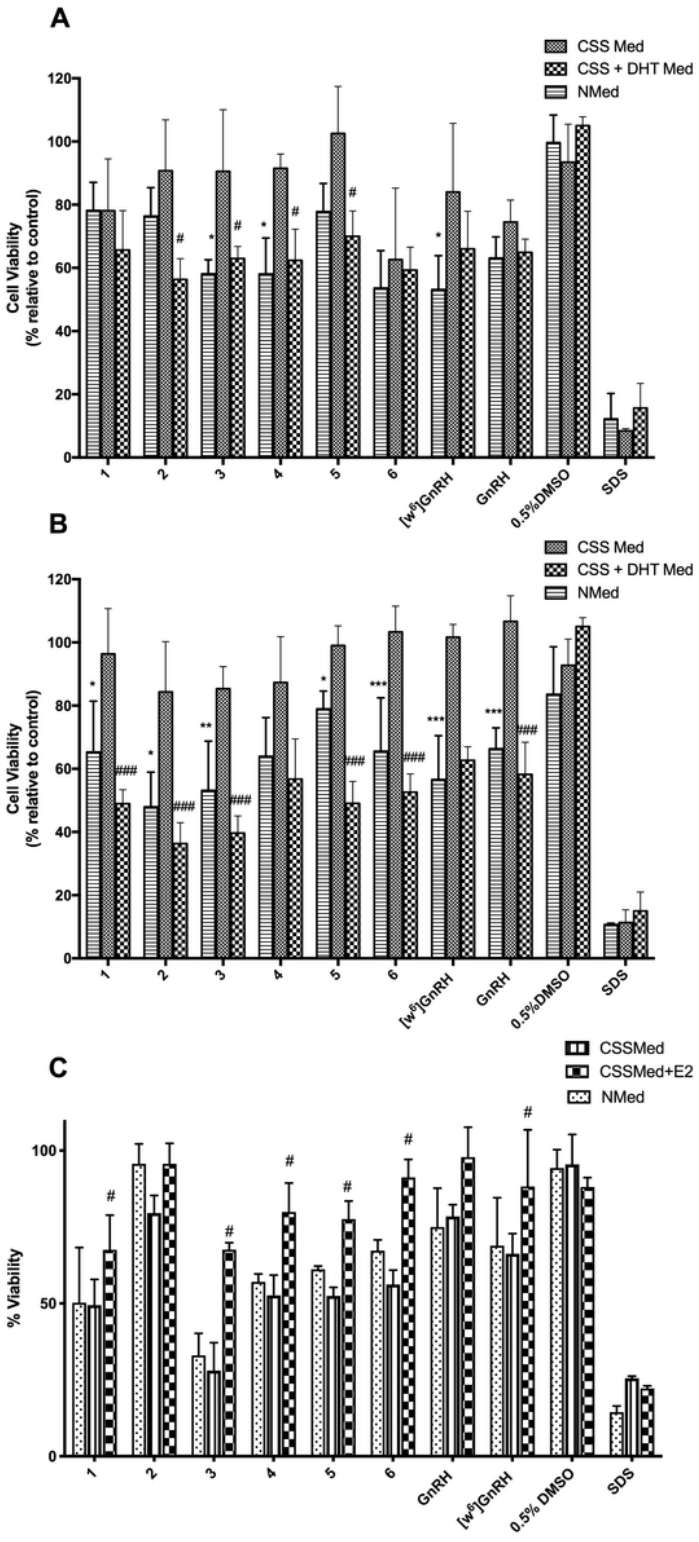

Figure 4

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 were 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. # $p < 0.05$, ### $p < 0.001$ for experiments performed in steroid reconstituted media in comparison with CSS and normal media

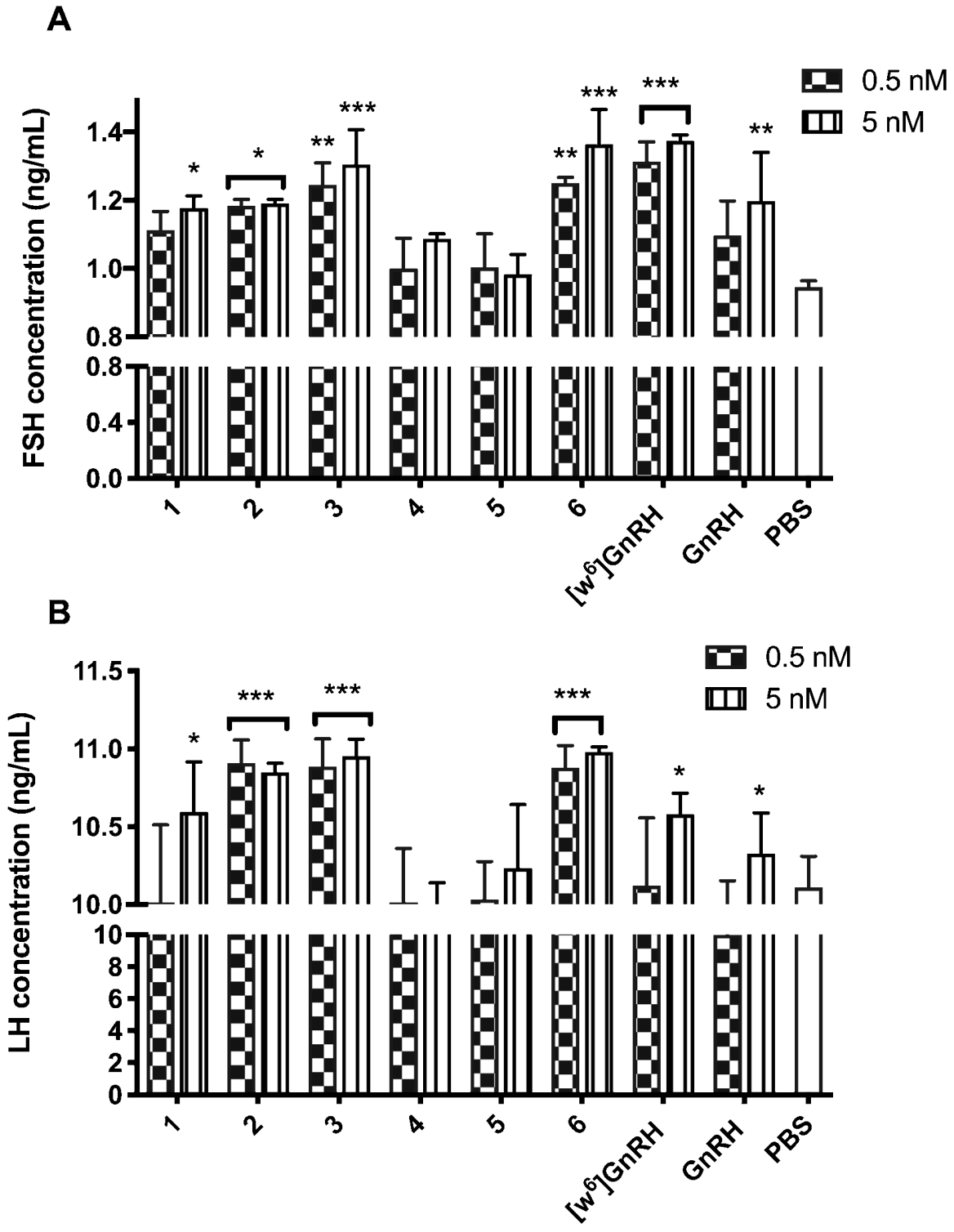

Figure 5

The effect of lipopeptides 1-6 and control peptides on the stimulation of the rat pituitary cells to release A) FSH and B) LH. Rat pituitary cells were dispersed and cultured in a media that contained different concentrations of peptide derivatives, 0.5, 5, and 50 nM. The level of LH and FSH was detected after 2 h incubation at 37 ºC using an ELISA kit. One-way ANOVA followed by Dunnett's post hoc test was used for statistical analysis. * $p<0.05$; **, $p< 0.01$, *** $p< 0.001$, increase in the FSH and LH level when compared to the PBS (negative control group)

Supplementary Files

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- 1.png