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New Gonadotropin-releasing Hormone Glycolipids with Direct Antiproliferative Activity and Gonadotropin-Releasing Potency

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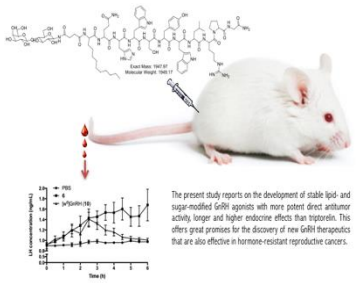
Graphical abstract



Gonadotropin-releasing hormone (GnRH) is an endogenous peptide with a short biological half-life. Although GnRH analogs (eg. triptorelin) are developed with enhanced stability compared to the native peptide, they still suffer from poor biological stability and pharmacokinetic properties. Furthermore, they are only effective in the treatment of hormone-dependent reproductive cancers. In this study we applied lipidation and glycosylation along with D-amino acid substitution at position 6 (D-Trp^6) to improve the stability, permeability, and consequently the potency of the GnRH peptide and triptorelin. We showed that the conjugation of GnRH with a lipid moiety and carbohydrates made all modified constructs (**1-8**) more stable than the parent peptide against enzymatic degradation (5.5 to 6.5 times). Two of the lactose-modified glycolipopeptides, **3** and **6**, showed 27 and 16 times higher membrane permeability than the parent GnRH, respectively. All analogs with D-Trp^6-substitution (**4-6**) exerted GnRH receptor-mediated antiproliferative activity in prostate and ovarian GnRH-receptor positive cell lines. They were more potent than triptorelin in the hormone-independent prostate cancer cell line: DU145. Compound **6** (lactose-modified) was the most potent analog for stimulating the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) gonadotropins from rat pituitary cells *in vitro*. The same glycolipopeptide exhibited a higher efficacy and duration of action in stimulating the release of LH than triptorelin in a preclinical mouse model. The superior activity

of lipid- and carbohydrate-substituted triptorelin analog **6** made it a promising candidate for the development of new GnRH agonists to treat both hormone-dependent and hormone-refractory prostate cancer.

.LIST OF ABBREVIATIONS

°C: degrees Celsius, ANOVA: Analysis of variance, AUC: Area under the curve, BBB: Blood brain barrier, Boc: tert-Butyloxycarbonyl, C12: 2-amino-D,L-dodecanoic acid, CSS: Charcoal stripped serum, DHT: Dihydrotestosterone, DMEM: Dulbecco's modified Eagle's medium, DMSO: Dimethyl sulfoxide, E2: 17ß-estradiol, FBS: Fetal bovine serum, FSH: Follicle-Stimulating Hormone, Gal: Galactose, Glc: Glucose, GnRH: Gonadotropin-Releasing Hormone, HPLC: High performance liquid chromatography, HR-MS: High resolution mass spectrometry, Lac: Lactose, LC-MS: Liquid Chromatography-Mass Spectrometry, LH: Luteinising-Hormone, MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), P_{app}: Apparent permeability, PBMC: Peripheral blood mononuclear cell, PBS: Phosphate-buffered saline, RP-HPLC: Reverse-phase-high performance liquid chromatography, Rt: Retention time, $t_{1/2}$: half-life

Keywords: Antitumor GnRH analogs; cancer therapy; hormone-refractory prostate cancer; peptide delivery; reproductive cancer; glycolipid.

1. INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a short-acting decapeptide with a biological half-life of less than 30 min (Chan and Nerenberg, 1987). It is secreted by the hypothalamus and stimulates the release of LH and FSH from the anterior pituitary gland. Structural modification of GnRH has resulted in analogs with higher receptor affinity and improved proteolytic stability than native GnRH. These agonists (e.g. triptorelin, buserelin, goserelin and leuprolide) are currently in clinical use (Berger et al., 1991). Continuous administration of GnRH agonists leads to a sustained GnRH receptor occupancy and desensitization of GnRH receptors in the pituitary, leading to medical castration (Pawson et al., 2008). Hormonal castration via the GnRH receptor is a well-established strategy for the treatment of hormone-dependent cancers of prostate, breast, ovarian and endometrial tissues (Schally et al., 1984).

A direct anticancer activity has also been reported for the GnRH analogs (Marelli et al., 2007). GnRH receptors are overexpressed both in reproductive organ cancers and cancers unrelated to the reproductive system (Franklin et al., 2003). GnRH analogs exert specific GnRH receptor-mediated antiproliferative, antimitogenic, and antimetastatic activities on cancer cells (Marelli et al., 2007). A direct relationship has been found between this effect of GnRH agonists and the level of GnRH receptor expression in the particular cell line.(Morgan et al., 2008)

Modifications that retard the dissociation rate of the GnRH analog from its receptor have been shown to increase its biopotency and bioavailability *in vivo*, which are highly dependent on the peptide's stability and permeability across biological membranes. In spite of extensive research in the field of hormonal therapy and development of various GnRH agonists over the past three decades, GnRH peptide analogs still suffer from poor pharmacokinetic properties. Depot formulations that are injected subcutaneously are also available; however, they can lead to several side effects such as leukocytoblastic vasculitis (Turk et al., 2007) and injection-site granulomas (Shiota et al., 2007). Subcutaneous implants have also been developed. Implants require a surgical incision and cause even more significant reactions at the administration site than depot formulations (Abouelfadel and Crawford, 2008). Hence, development

of a more stable GnRH agonist with improved pharmacokinetics that allows for less frequent administration is highly desirable. Different strategies have been investigated for the effective delivery of GnRH agonists to enhance stability and improve their transport profile in biological systems (Mezo et al., 2008).

Several chemical modifications have been applied to improve the biological and physicochemical properties of peptides. Conjugation to carbohydrates has been shown to considerably enhance peptide stability, permeation across biological membranes, and bioavailability (Egleton and Davis, 2005; Varamini et al., 2012b). Lipidation increases the lipophilicity and membrane-like properties of the peptides, thus facilitates their penetration across biological barriers (Griffin, 2011). Lipoamino acids are α-amino acids with alkyl side chains of varying length (usually C8-20). Their similarity to α-amino acids allows facile incorporation into the structure of peptides (Toth, 1994). Lipoamino acids were shown to be cleaved from their parent peptides *in vitro* (Blanchfield et al., 2005) thus can be incorporated into peptide sequences do develop a more lipophilic prodrug. Use of both lipidation and glycosylation will confer a balance between the lipophilic and hydrophilic characteristics. This may result in higher potency by improving peptide stability against enzymatic hydrolysis and permeability through biological membranes (Drouillat et al., 1998; Koda et al., 2008; Nomoto et al., 1998).

Tyr^5-Gly^6 and Pro^9-Gly^{10} bonds, and to a lesser extent, the $pGlu^1$-His^2 and Gly^6-Leu^7 bonds are known as the main cleavage sites of GnRH (Griffiths and Mcdermott, 1983). Therefore, due to their susceptibility, these bonds are the most common modification sites to stabilize the GnRH peptide. The majority of clinically used GnRH agonists (including triptorelin), contain a D-amino in place of glycine at position 6 (Beyer et al., 2011). This replacement leads to a greater metabolic stability of the compound (Karten and Rivier, 1986). Furthermore, the D-amino acid in this position enhances receptor binding by stabilizing the type II ß-turn conformation that has been shown to be important for interaction with the receptor (Laimou et al., 2010). Thus the GnRH agonists with D-amino acid modification at position 6 were more potent than the GnRH peptide itself. There are also reports on the development of active agonists by D-amino acid substitutions at position 7 (Ling and Vale, 1975).

In this study we applied a combination of glycosylation, lipidation and D-amino acid substitution strategies to improve the stability and potency of the GnRH peptide. We hypothesized that increased stability and potency would lead to GnRH analogs with better antitumor activity. This would provide an added benefit for application in hormone-dependent cancers and make the new agonist(s) also suitable for application in hormone-independent cancers of e.g. prostate and ovary.

Three different types of carbohydrates: glucose, galactose and lactose, were attached to the N-terminus of the GnRH peptide analogs. C12 (2-Amino-D,L-dodecanoic acid) was conjugated either to the N-terminus of the glycopeptides (compounds **1**-**6**) or incorporated into the middle of the sequence at position 7 in place of Leu (compounds **7**-**8**). D-Amino acid substitution at position 6 was performed to produce derivatives of triptorelin ([w^6]GnRH). Glutamine (Gln) was used in place of glutamic acid (Glu) as the first amino acid in the GnRH sequence in compounds **1-8** for two reasons: firstly, it is known that endogenous GnRH (**9**) is produced with N-terminal glutamine which spontaneously cyclizes to form pyroglutamic acid, and secondly we have previously shown that GnRH conjugates with Gln have longer half-lives than those with Glu (Moradi et al., 2013).

Constructs **1-8** were tested *in vitro* to assess their permeability through biological membranes and stability against enzymatic degradation. The compounds' direct antiproliferative activity in human prostate and ovarian cancer cell lines and their toxicity to normal blood and pituitary cells was investigated along with their ability to stimulate the release of gonadotropins from pituitary cells. Additionally, the impact of sex steroids (dihydrotestosterone, DHT, or 17ß-estradiol, E2) on the growth inhibitory effects of the GnRH analogs was studied. The lead compound (**6**) was selected from *in vitro* studies and, along with triptorelin (**10**) as a positive control, was tested in Swiss albino mice to investigate its ability to stimulate the release of LH after subcutaneous administration.

2. MATERIALS AND METHOD

2.1. Design and synthesis

2.1.1. Carbohydrate synthesis

Acetylated, butanoic acid-conjugated carbohydrates were synthesized adapting or following published procedures (Varamini et al., 2012b) as summarized in Scheme 1, and ^{1}H-NMR spectra matched the published ones (Moradi et al., 2013). Chemical structures are shown in Table 1.

2.1.2. Peptide synthesis

Peptides were assembled on Rink amide resin following the *in situ* neutralization protocol for Fmoc solid-phase chemistry.(Alewood et al., 1997) Briefly, Nα-Fmoc-protected amino acids (4.2eq.) were activated with HBTU/DIPEA (4eq./5eq.) and double coupled for at least 45 minutes each. The following side chain protecting groups were used: Arg(Pbf), Tyr(tBu), Ser(tBu), Trp(Boc), His(Trt), Gln(tBu). The Fmoc protecting group was removed by treatment with piperidine in DMF (1:5, 1 x 10 min, 1 x 20 min). The Dde-protected C12-lipoamino acid was synthesized following published procedures.(Gibbons et al., 1990; Ross et al., 2008) The Dde protecting group was removed by treatment with hydrazine hydrate in DMF (1:50, 3 x 5 min). Carbohydrate derivatives 1**3a-c** (1.3eq.) were activated with HBTU/DIPEA (1.25eq./2eq.) and coupled overnight. The resin was washed with dimethylformamide, dichloromethane and methanol and treated with hydrazine hydrate/methanol (3:1, 2 x 30 min) to remove the acetyl protecting groups. The resin was washed thoroughly with methanol and dried under vacuum overnight. The peptide was cleaved by treatment with a mixture of TFA/water/tri-isopropylsilane (95:2.5:2.5) for 2 hours. Addition of cold diethyl ether precipitated the peptide, the peptide suspension was centrifuged and the supernatant discarded. The peptide pellet was washed with cold diethyl ether once and then dissolved in a mixture of acetonitrile and water containing 0.1% trifluoroacetic acid and lyophilized.

2.1.3. Compound Purification

Peptides were purified by reverse phase high performance liquid chromatography (RP-HPLC) on a Shimadzu system using a Vydac C18 column (5µm, 22 x 250 mm) running a gradient of two solvents, A: H_2O, 0.1% TFA, and B: acetonitrile/H_2O 9:1, 0.1% TFA. Either a gradient of 20% to 60% B over 60 minutes (peptides **1-3**, **7,** and **9-10**) or a gradient of 10% to 60% B over 70 minutes (peptides **4-6** and **8**) was used at a flow rate of 10 ml/min. Collected fractions were analyzed by High resolution MS and ESI-MS and analytical RP-HPLC using a Vydac C4 and C18 column (5µm, 4.6 x 250mm) and a gradient of 0% to 100% B over 30 minutes at a flow rate of 1 ml/min. Pure fractions were combined and lyophilized.

2.2. Characterization

1. Glc-C12-[Q^1]GnRH; HPLC retention time: C4 Vydac column (17.7/17.9 min), C18 Vydac column (18.5/18.7 min); HR-MS $[M+2H]^{2+}$: calc.: 829.4385, found: 829.4421.

2. Gal-C12-[Q^1]GnRH. HPLC retention time: C4 Vydac column (17.0/17.4 min), C18 Vydac column (17.7/18.2 min); HR-MS $[M+2H]^{2+}$: calc.: 829.4385, found: 829.4425.

3. Lac-C12-[Q^1]GnRH. HPLC retention time: C4 Vydac column (16.8/17.2 min), C18 Vydac column (17.5/17.9 min); HR-MS $[M+2H]^{2+}$: calc.: 910.4649, found: 910.4691.

4. Glc-C12-[Q^1][w^6]GnRH. HPLC retention time: C4 Vydac column (18.3/18.7 min), C18 Vydac column (18.9/19.1 min); HR-MS $[M+2H]^{2+}$: calc.: 893.9674, found: 893.9874.

5. Gal-C12-[Q^1] [w^6]GnRH. HPLC retention time: C4 Vydac column (18.9/19.3 min), C18 Vydac column (18.6/19.1 min); HR-MS $[M+2H]^{2+}$: calc.: 893.9674, found: 893.9712.

6. Lac-C12-[Q^1] [w^6]GnRH. HPLC retention time: C4 Vydac column (18.0/18.4 min), C18 Vydac column (19.8/20.4 min); HR-MS $[M+2H]^{2+}$: calc.: 974.9938, found: 974.9981.

7. Lac-[Q^1][w^6][$C12^7$]GnRH. HPLC retention time: C4 Vydac column (17.0/17.1 min), C18 Vydac column (18.0 min); HR-MS $[M+2H]^{2+}$: calc.: 918.4518, found: 918.4545.

8. Lac-[Q^1][$C12^7$]GnRH. HPLC retention time: C4 Vydac column (18.2/18.7 min), C18 Vydac column (19.1/19.8 min); HR-MS $[M+2H]^{2+}$: calc.: 853.9229, found: 853.9261.

2.3. In vitro experiments

2.3.1 Cell lines

The Caco-2 human epithelial colorectal adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, USA). Different steroid hormone-dependent and independent, sex-specific human carcinoma cell lines were used. The LNCaP (GnRH receptor positive; androgen-sensitive prostate adenocarcinoma), PC3 and DU145 (GnRH receptor positive; androgen-independent human carcinoma), OVCAR-3 (GnRH receptor positive; estrogen, progesterone and androgen-receptor positive ovarian carcinoma) and SKOV-3 (GnRH receptor negative; estrogen-resistant ovarian carcinoma) human cell lines were purchased from ATCC and were provided by Professor Judith Clements at the Translational Research Institute and Queensland University of Technology, and Professor Rodney Minchin, School of Biomedical Sciences, The University of Queensland.

The method for Caco-2 cell homogenate stability and membrane permeability is described in Supplementary Information

2.3.2 Tumor cell proliferation assay (MTT assay)

MTT cell proliferation assay was performed as previously described.(Moradi et al., 2015b) The cells were passaged and plated (90 µL/well) in flat-bottomed 96-well plates at 2×10^5 cells/mL for LNCaP, DU145 and OVCAR-3 or 1×10^5 cells/mL for PC3 and SKOV-3 cells. Compounds were used at a final concentration of 1, 10, 25, 50 or 100 µM in 0.5% DMSO in the culture media (n = 3/compound, in at least 3 independent experiments).

2.3.3 Triptorelin competition assay

LNCaP, DU145 and OVCAR-3 cells were pre-treated with 100 µM (100 µL) of triptorelin. After 2 h incubation, cells were washed and fresh media added at 90 µL. Compounds were added to each well (10 µL) at their effective concentration (50 µM) and incubated for 48 h followed by MTT assay.

2.3.4 Steroid treatment studies

After cells reached 70% confluence, they were washed twice with PBS and media that contained 10% charcoal stripped FBS (CSS) was added to the flask. Cells were either seeded in 96-well plates after 48 h incubation and treated with compounds **1-8** and controls at 50 µM to perform the MTT assay or were treated with fresh CSS media that contained 5 nM 17ß-estradiol (E2) or 50 nM dihydrotestosteron (DHT) for an additional 48 h incubation. Treated cells were plated in 96-well plates. Compounds were added at 50 µM. MTT assay was performed after 48 h incubation.

2.3.5 Isolation of PBMCs

This assay was completed following approval from the University of Queensland Ethics Committee (Ethics Approval Number: 2009000661). Blood samples (4 mL) were taken from a healthy adult volunteer and PBMCs were isolated

by Ficoll gradient centrifugation at 400 g for 30 min. Mononuclear cell layer was retained at the plasma-Ficoll interface and washed with RPMI 1640 (x3). Cells were resuspended in 10% FBS in RPMI and seeded at 1×10^6 cells/mL (80 µL) in 96-well flat bottom plates. Phytohemagglutinin (10 µg/mL) was added to activate the cells. After 1 h incubation at 37 °C in a 5% CO_2 atmosphere, compounds **1-10** in 5% DMSO in PBS were added (10 µL/well) at 50 and 100 µM. The MTT assay was performed after 48 h incubation as above.

2.3.6 In vitro LH and FSH release assays

2.3.6.1 Rat pituitary cell preparation

Pituitary cells were isolated and cultured according to a previously published method.(Moradi et al., 2015b) Cells were plated at 4×10^5 cells/mL in 96-well plates at a density of 3×10^4 cells/well and incubated for 72 h at 37 ºC.

2.3.6.2 LH and FSH release assay

Pituitary cell dispersion was performed as described elsewhere.(Moradi et al., 2015a) Briefly, plated pituitary cells were centrifuged at 400 g for 10 min. Cells were washed and replaced by challenge media, which contained 0.1% bovine serum albumin in DMEM. GnRH derivatives **1-8** and control peptides (GnRH and triptorelin) were then added at 0.5, 5 or 50 nM (10 µL). The treated pituitary cells were incubated at 37 ºC for 2 h. The level of LH and FSH was quantified according to the manufacturer's instructions using a commercial enzyme-linked immunosorbent assay (ELISA) kit (USCN Life Science Inc., Wuhan, China).

2.4. In vivo experiments

2.4.1 Animals and compounds' administration

Male Swiss Albino mice (6-8 weeks of age, weighing 34-45 g at the time of assessment) were purchased from The University of Queensland biological resources breeding facility. Mice were housed five per cage in an artificially lit room on a 12 h light/12 h dark cycle at controlled temperature (22.2 ± 0.2 °C; mean ± StDev) and humidity (51–65%) with food and water available *ad libitum*. The animal experimentation protocols were approved by The University of Queensland Animal Ethics Committee (AEC#SCMB/005/11/ARC) and performed according to NHMRC animal handling guidelines.

Prior to start of the experiments, mice were allowed to acclimatize to the new environment for two weeks to minimize stress and thus experimental variation. During this period, mice were trained every alternative day by mimicking experimental manipulation procedures.

Each mouse was administered 50 µmol of compounds **6**, triptorelin or vehicle (10% DMSO) subcutaneously (50 µL). Just prior to administering the compounds, a single tail tip blood sample was collected to establish baseline, adhering to guidelines established previously (Steyn et al., 2013). Post administration, 2 µL of blood was collected at 30-minute intervals for duration of 6 hours. Collected blood was diluted in 58 µL of PBS-T and immediately stored in dry ice. Samples were kept at -80 °C prior to batch analysis using a validated ultra-sensitive mouse LH ELISA method (Steyn et al., 2013).

2.4.2 Assessment of in vivo LH secretion

A sensitive sandwich ELISA was used to measure serum concentration of LH, strictly adhering to published methodology (Steyn et al., 2013). Briefly, a monoclonal antibody (50 µL anti-bovine LH beta subunit, University of California) was used to coat 96-well high-affinity binding microplates and incubated overnight at 4°C. Using a 2-fold serial dilution of mouse LH in 0.2% (w/v) BSA-1×PBS-T (PBS with 0.05% Tween 20), a standard curve was

generated (0.00195 to 4 ng/mL). Wells were incubated with 200 µL of blocking buffer (5% skim milk in PBS-T) for 2 h at room temperature (RT). Detection antibody (polyclonal antibody, rabbit LH antiserum, 50 µL) along with the LH standards and plasma samples were incubated for 1.5 h at a final dilution of 1:10000 at RT. This step was followed by the addition of 50 µL horseradish peroxidase-conjugated antibody (polyclonal goat anti-rabbit antibody) at a final dilution of 1:2000 and 1.5 h incubation at RT. O-phenylenediamine substrate (containing 0.1% H_2O_2) was added to each well and left at RT for 30 minutes. Hydrochloric acid at 3 M concentration was used to stop the reaction. The absorbance of each well was read at a wavelength of 490 nm (Sunrise; Tecan Group). OD values of unknowns were interpolated against a nonlinear regression of the LH standard curve and from there the LH concentrations were determined in the whole blood samples. LH secretory responses were also expressed as the area under the curve (AUC). The within and between assay coefficient of variation of LH assays were below 5%.

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2.5. Statistical analysis

Data are presented as means ± StDev. Differences between groups were identified by a one-way ANOVA followed by Dunnett's post-hoc test and comparison with the corresponding control group. All measures were performed using GraphPad Prism (version 6.0; Graph-Pad Software Inc.). The threshold level for statistical significance was set at $P<0.05$.

3. RESULTS AND DISCUSSION

We developed GnRH analogs that were more stable and potent than the currently available agonists. Improve stability of the GnRH peptide will increase its duration of activity, consequently reducing its dosing frequency.

The current GnRH analogs in clinical use achieve the desired pharmacological effect by primarily acting on the pituitary with less direct antiproliferative effects on tumor cells. We hypothesized that increasing the stability and potency of the GnRH agonists through conjugation of a lipid chain and carbohydrates might increase their direct antiproliferative activity (Hollosy et al., 2002). This would result in analogs with dual action in hormone-dependent cancers, both blocking the release of sex hormones and directly inhibiting the growth of the tumor. If the antitumor activity is strong, they can also be used in hormone-independent reproductive cancers.

3.1. Design and synthesis of GnRH and its derivatives

The native GnRH (**9**), triptorelin (**10**) and their glycolipid analogs **1**-**8** were synthesized, purified to a single peak (> 95% purity) by analytical RP-HPLC and characterized using high resolution mass spectrometry (HR-MS). All chemical structures and compounds' characterizations including HPLC and HR-MS figures are reported in the Supplementary Information (Table S1 and Figure S1). Modifications included the conjugation of lipid and carbohydrate moieties in GnRH-based analogs at position 1 (compounds **1-3)** or position 7 (compound **7**). Same modifications at position 1 and 6 were performed using a D-Trp^6-substituted peptide (triptorelin) yielding three triptorelin or [w^6]GnRH-based compounds (**4-6** and **8**). The amino acid sequences are shown in Table 2.

3.2. In vitro experiments

3.2.1. Caco-2 cell homogenate stability and membrane permeability assay

The conventional colorectal adenocarcinoma (Caco-2) cell homogenate assay was used to determine the effect of lipid and sugar conjugation on the stability of GnRH peptide *in vitro*. We applied lipidation and glycosylation techniques with a history of improving stability and membrane permeability to produce more effective GnRH analogs (Varamini et al., 2012a, b). The native GnRH peptide (**9**) had a half-life of around 10 min after incubation with Caco-2 cell homogenates (Table 3). The combination of lipidation and glycosylation caused a 4–6-fold increase (39.8–61.3 min) in the metabolic stability of the native peptide (**9**) regardless of the position of the lipid in the sequence. The same modification strategies in compounds with D-Trp at position 6 ([w^6]GnRH-based derivatives) and the lipid moiety at the N-terminus (**4–6**) significantly increased the half-life of the parent peptide (**10**) by 5.5–6.5-fold (p<0.05). Among all different carbohydrates used, lactose produced the most stable analogs in both GnRH- or [w^6]GnRH-based derivatives. The stability of GnRH glycolipid that bore the lipid modification in the middle of the sequence (**7**) was not further increased by D-Trp^6 substitution (**8**). The findings of the Caco-2 cell stability assay suggested that the best modification strategy was N-terminal lipid and sugar conjugation in [w^6]GnRH-based derivatives (**4–6**).

Although the pituitary gland sits outside the blood-brain barrier (Nussey and Whitehead, 2001), the GnRH agonist needs to cross cell membranes to reach blood circulation if administered via any route other than intravenous injection. Peptide analogs with a higher apparent permeability are shown to have better absorption and produce higher plasma peak concentrations compared to those with lower membrane permeability (Varamini et al., 2012b). A Caco-2 cell membrane model was used as a preliminary tool to screen membrane permeability. Propranolol was used as a positive control because it was completely absorbed by the gastrointestinal tract (GI)(Johnsson and Regardh, 1976). For propranolol, P_{app} was obtained at 0.8 and 1.3 × 10^{-5} cm/s in the first and second experiment, respectively. Mannitol was used in this experiment as a negative control due to very low absorption in the GI. The parent peptide, GnRH (**9**), displayed a low P_{app} of 1.2 × 10^{-7} cm/s. With a slight improvement, the permeability of triptorelin (**10)** was found to be 2.8 × 10^{-7} cm/s. However, conjugation of a lactose moiety to the peptides with N-terminal lipid, resulted in a significant increase in the apparent permeability of the peptides. Thus, compounds **3** and **6** (P_{app} of 32.8 and 19.2 × 10^{-7} cm/s, respectively) showed highest apparent permeability, when compared to GnRH, [w^6]GnRH and even the lactose-modified peptides with the lipid in the middle of the sequence, i.e. **7** and **8** (p< 0.05, Table 3).

These findings were in line with our previous report where the lactose group alone was shown to be the most effective carbohydrate moiety in improving the absorption of GnRH peptide through biological membranes (Moradi et al., 2014). It caused an unprecedented 700-fold increase in membrane permeability of a different peptide (Varamini et al., 2012b).

3.2.2. Tumor cell proliferation assay (MTT assay)

GnRH pathways have been shown to be involved in cell growth, invasion and angiogenesis of peripheral tumors, to varying extent (Skinner et al., 2009). GnRH receptor ligands have been shown to act differently on the GnRH receptor positive peripheral tissues at different doses (Mezo and Manea, 2010); a dose-dependent increase in cell proliferation at low (nanomolar) concentrations and inhibitory action at higher (micromolar) concentrations (Chen et al., 2007). This has been explained by the differential expression profiles of GnRH receptors (Mezo and Manea, 2010). Therefore, in this study tumor cell lines with a variety of GnRH receptor expression levels and hormone dependence were used to investigate this effect of the GnRH glycolipids. Cell proliferation was evaluated by assessing the mitochondrial reduction of MTT in three GnRH-receptor positive prostate cancer cell lines (LNCaP, DU145 and PC3) and ovarian cancer cell lines with (OVCAR-3) and without (SKOV-3) the GnRH-receptor.

- Prostate cancer cell lines: All analogs **1**-**8** inhibited the growth of hormone DU145 cells after 48 h incubation with IC_{50} values between 36–62 μM (Table 4). The most potent analogs were [w^6]GnRH-based derivatives that bore a sugar and lipid at their N-terminus (**4**, **5** and **6**) with IC_{50} values obtained at 39.4, 37.4 and 36.4 μM, respectively (p<0.05). Compounds **4-6** were ~1.7 times more potent than triptorelin, indicating that sugar and lipid modification could improve their direct antitumor activity. This finding is of particular importance for treating prostate cancers that progress to hormone-refractory state. Although compound **8** had the same sugar (lactose) and D-Trp modification as compound **6**, replacement of Leu with C12 at position 7 reduced its activity against DU145 cells by about 50%. GnRH peptide did not affect the growth of DU145 cell line (IC_{50} >100 μM). A similar pattern, albeit with slightly higher IC_{50} values, was obtained when the other GnRH receptor positive prostate cancer cell line, LNCaP. Analogs **5** and **6** produced a significant inhibitory effect on the growth of LNCaP prostate cancer cells (IC_{50} values at 57.6 μM for analog **5** and 52.2 μM for analog **6**). This effect was higher than that of triptorelin (p<0.05, Table 4). The above finding suggested that N-terminal lipid and sugar modification, regardless of the type of sugar, produced the most potent analogs against DU145 and LNCaP cell lines with direct growth inhibitory activity. It has previously been reported that the position of a bulky residue on the N-terminus of the GnRH backbone enhanced the conformational stability of the peptide and forced the formation of a β-II turn for greater receptor binding. This in turn enhanced GnRH receptor-mediated antiproliferative activity.(Pfleger et al., 2002) It is plausible the position of bulky carbohydrate and lipoamino acid residues on the N-terminus of the GnRH backbone contributed to the enhanced the antitumor and gonadotropin release activity of GnRH glycolipids. The growth of PC3 cells was not significantly inhibited by any of the analogs **1**-**8** or controls **9**-**10** (Table 4). This finding was in line with a previous report where PC3 cell growth was not inhibited by triptorelin, though triptorelin significantly inhibited the growth of LNCaP cells (Ravenna et al., 2000).

- Ovarian cancer cell lines: The growth of OVCAR-3 cells was affected to a variable extent after being treated by different glycolipopeptides. GnRH-based derivatives, **1** and **2**, with a glucose or galactose moiety produced the highest antiproliferative activity with IC_{50} values at 41.8 and 46.3 μM, respectively. Nevertheless, lactose-modified GnRH-based compounds **3** and **7** did not exert any effects on these cells. Analogs **4-6** in the [w^6]GnRH-based group of compounds significantly inhibited the growth of OVCAR-3 cells (IC_{50}: 60–65 μM) while compound **8** in the same

group did not produce any significant effect. The absence of growth inhibitory effects by compounds **7** and **8** against the OVCAR-3 cell line can be explained by the mid-sequence lipid position. It is possible that mid-sequence lipid modification renders the compounds unable to activate the relevant signaling pathway and eventually to inhibit the cell growth.

No significant growth inhibition was observed when the GnRH-receptor negative SKOV-3 cells were treated with compounds **1-10** (Table 4).

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

PBMCs as a model of non-cancerous human cells and rat pituitary cells were used to determine the toxicity of compounds **1-8**. MTT assay was also used to determine the effect of the GnRH derivatives on the proliferation of non-cancerous cells: human PBMCs and isolated rat pituitary cells. No adverse effects were observed in the growth of pituitary cells or PBMCs cells after being treated with the two highest concentrations of the GnRH derivatives **1-8** . (Figure 1, $p>0.05$), suggesting that the growth inhibitory effect was selective for cells that overexpressed the GnRH-receptor.

3.2.4. Triptorelin competition studies: GnRH receptor-mediated antiproliferative activity

In order to investigate whether the antiproliferative activity of glycolipopeptides was mediated through GnRH receptors, a competitive binding study was performed in DU145, LNCaP and OVCAR-3 cell lines. The effect of all compounds with significant growth inhibitory activity (at 50 μM) was antagonized after 2 h pretreatment with the 100 μM triptorelin ($p<0.05$, Figure 3a-c. Agonist pretreatment did not affect the activity of analogs with low antiproliferative activity on these cell lines (analogs **2-3** and **7-8** in LNCaP and compounds **3** and **7-8** in OVCAR-3 cells). These results suggest a selective receptor-mediated action of the sugar- and lipid-modified GnRH analogs in receptor-positive cell lines. It has previously been reported that the receptor-mediated antiproliferative activity of GnRH analogs occurred by inhibiting the mitogenic signal transduction pathways of the epidermal growth factor receptor in prostate, endometrial, ovarian, and breast cancer cell lines.(Grundker et al., 2000) This could explain the antiproliferative effect of GnRH analogs on GnRH-R positive cell lines (LNCaP, OVCAR-3 and DU-145) and not on GnRH receptor negative cells (SKOV-3).

3.2.5. Impact of sex steroids on the antiproliferative activity

Steroid hormones were previously shown to have regulatory functions on the growth inhibitory effect of GnRH agonists and the expression level of GnRH receptors, regardless of cells being hormone-dependent or not (Kang et al., 2001; Leuschner et al., 2003). Herein, we studied the correlation between the antiproliferative activity of glycolipopeptides and steroids in a steroid-depleted media (CSS). The antiproliferative effect of GnRH glycolipids (50 μM) was investigated in the presence or absence of DHT and E2 in DU145 and OVCAR-3 cells, respectively. The sensitivity of DU145 cells to the growth inhibitory effect of compounds **1-10** was significantly reduced (30-41%, $p<0.05$) when cultured in steroid-depleted media (CSS). However, after reconstitution of the media with DHT, the antiproliferative activity of the compounds was restored (Figure 2a). These results were consistent with previous reports that showed a steroid-dependent antiproliferative activity of GnRH agonists.(Leuschner et al., 2003) In this study, Leuschner et al. reported that DHT-dependent upregulation of GnRH receptor expression (up to 119%) in GnRH receptor positive prostate cancer cells was responsible for this hypersensitivity (Angelucci et al., 2009). Receptor upregulation was observed to the same extent when cells were treated with a GnRH agonist and DHT

(Angelucci et al., 2009). The higher sensitivity of DU145 cells to glycolipopeptides and triptorelin is plausibly due to the increased number of GnRH receptors in the presence of DHT.

In contrast to DU145 cells, the antiproliferative effects of compounds on OVCAR-3 cells was either unaffected (**1-5** and **9-10**) or increased (**6-8**) in CSS media. However, the reconstitution of the media with E2 significantly decreased the sensitivity of the OVCAR-3 cells to all compounds **1-10** compared to the CSS media. (Figure 2b). It has been previously shown that estrogen functions as a mitogen in OVCAR-3 cells, which can neutralize the growth inhibitory effect of GnRH-based compounds (Kang et al., 2001). This observation was consistent with a previous study where pre- or co-treatment with E2 significantly attenuated the growth inhibitory effect of a GnRH agonist. In this report, a down-regulation of GnRH receptor mRNA was observed, which could explain the attenuation of growth inhibition after treatment with 17ß-estradiol (Kang et al., 2001).

3.2.6. In vitro LH and FSH release study

Gonadotropin release was studied using rat anterior pituitary cells in primary culture after 2 h incubation with the analogs (Figure 4a). Compounds **5** and **6** increased the level of FSH up to 1.8 times from dispersed pituitary cells compared to the negative control (up to 2 ng/mL for compound **6** compared to 1.1 ng/mL for PBS, $p>0.05$). GnRH (**9**) and triptorelin (**10**) also stimulated the FSH release significantly up to 1.8 and 2.3 ng/mL, respectively ($p>0.05$).

The level of LH release was significantly increased by most of the glycolipopeptides (**1**, **3-6** and **8**, Figure 4b, $p<0.05$). However, the stimulatory effects of [w^6]GnRH-based compounds **4-6** with the N-terminal lipid were higher than other analogs (2.5 to 3.8 times relative to PBS control). Compounds **5** and **6** were effective in stimulating the LH secretion level in culture at all three concentrations (up to 173 ng/mL for compound **6** compared to 47 ng/mL for the negative control, $p<0.05$). The effect of compound **6** at 50 nM was even higher than triptorelin and GnRH at the same concentration ($p<0.05$). The higher efficacy of [w^6]GnRH-based glycolipids (**4-6**) could be due to their higher stability, allowing them to stay intact for longer after binding to receptors on the pituitary.

3.3. In vivo experiment: Efficacy studies for the release of LH in mice

Analog **6** showed the most promising results in the *in vitro* studies. Thus, it was selected as the lead compound for further testing in mice. A preclinical method was developed in Swiss Albino mice in the context of our objectives. This mouse model was particularly valuable because: (1) the human GnRH receptor is homologous to the mouse receptor (Millar et al., 2004); (2) GnRH receptor agonism was determined by measuring the level of LH after acute administration and over a course of 6 hours post-injection; (3) frequent sampling allowed discrimination of the natural pulsatile effect on the release of LH from the effect of the compounds. Although it is critically important, the majority of studies that aim to develop new GnRH analogs do not consider endogenous LH release patterning; (4) a robust sensitive methodology that has been developed by Steyn et al. (Steyn et al., 2013) was used for the first time to measure the level of LH release to determine the efficacy of GnRH receptor ligands. Altogether this methodology provided a distinctive approach to gain insight into the agonist/antagonist efficacy of GnRH receptor ligands over a course of six hours post-treatment.

LH release was measured every 30 min, over 6 h after SC administration of compounds **6**, triptorelin (as a positive control) and PBS as the negative control. Plasma LH level rose gradually after administration of both analog **6** and triptorelin, with a peak response for triptorelin at 2.5 h (1.4 ng/mL). However, the LH level continued to rise after administration of compound **6** beyond the peak level observed for triptorelin giving an LH concentration of 1.7 ng/mL after 6 h of treatment (Figure 5a). These results indicated that compound **6** had a long-lasting effect with a higher maximum plasma level and hence, a better potency than triptorelin (due to the stronger stimulation of LH release at the same dose). In contrast to compound **6**, the effect of triptorelin started to decline after 2.5 h

The area under curves (AUC) of LH release was obtained over 6 h indicating the duration of action of the compounds. A significant increase from 0.3 to 2.7 ng/mL/6h was observed in mice treated with glycolipopeptide **6** compared to the PBS group (Figure 5b, $p<0.05$). The AUC (duration of action) of LH released by mice treated with triptorelin was measured at 1.3 ng/mL/6h, which was significantly less than triptorelin ($p<0.05$). The *in vivo* studies suggest that compound **6** showed an improved potency and duration of action compared to triptorelin.

CONCLUSION

GnRH agonist therapy is a powerful therapeutic approach for the treatment of many conditions in reproductive medicine, particularly hormone-dependent tumors (e.g., prostate, breast, endometrial, and ovarian cancers). By exerting a direct and selective antiproliferative activity through GnRH receptor in overexpressing tumor cell lines, [w^6]GnRH-based glycolypopeptides (**4-6)** offer great promises for the discovery of new GnRH therapeutics that are effective in hormone-independent prostate cancer. In particular, glycolipopeptide **6** showed the highest *in vitro* metabolic stability, direct antiproliferative activity, LH release efficacy in pituitary cells, and potent and long-acting gonadotropin releasing function. Compound **6** was superior to triptorelin in all *in vitro* and *in vivo* examinations, making it a promising candidate for the development of new GnRH agonists to treat hormone sensitive and refractory prostate cancer.

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SUPPLEMENTARY MATERIAL

All general materials and instruments are described in Supplementary Information. The general reagents and instruments, details of Caco-2 cell homogenate stability assay and Caco-2 cell permeability assay are described in supplementary information. Table S1 represents the chemical structure of compounds **1-8,** and figure S1 shows HPLC and HR-MS characterizations for all compounds **1-10.**

REFERENCES

Abouelfadel, Z., Crawford, E.D., 2008. Leuprorelin depot injection: patient considerations in the management of prostatic cancer. Therapeutics and clinical risk management 4, 513-526.

Alewood, P., Alewood, D., Miranda, L., Love, S., Meutermans, W., Wilson, D., 1997. [2] Rapid in situ neutralization protocols for Boc and Fmoc solid-phase chemistries, in: Gregg, B.F. (Ed.), Methods Enzymol. Academic Press, pp. 14-29.

Angelucci, C., Lama, G., Iacopino, F., Ferracuti, S., Bono, A.V., Millar, R.P., Sica, G., 2009. GnRH receptor expression in human prostate cancer cells is affected by hormones and growth factors. Endocrine 36, 87-97.

Berger, H., Heinrich, N., Albrecht, E., Kertscher, U., Oehlke, J., Bienert, M., Schafer, H., Baeger, I., Mehlis, B., 1991. Gonadotropin-Releasing-Hormone (Gnrh) Analogs - Relationship between Their Structure, Proteolytic Inactivation and Pharmacokinetics in Rats. Regulatory peptides 33, 299-311.

Beyer, D.A., Amari, F., Thill, M., Schultze-Mosgau, A., Al-Hasani, S., Diedrich, K., Griesinger, G., 2011. Emerging gonadotropin-releasing hormone agonists. Expert Opin Emerg Dr 16, 323-340.

Blanchfield, J.T., Lew, R.A., Smith, A.I., Toth, I., 2005. The stability of lipidic analogues of GnRH in plasma and kidney preparations: the stereoselective release of the parent peptide. Bioorganic and Medicinal Chemistry Letters 15, 1609-1612.

Chan, R.L., Nerenberg, C.A., 1987. Pharmacokinetics and Metabolism of LHRH Analogs, in: Vickery, B.H., Nestor, J.J., Jr. (Eds.), LHRH and Its Analogs. Springer Netherlands, pp. 577-593.

Chen, C.L., Cheung, L.W., Lau, M.T., Choi, J.H., Auersperg, N., Wang, H.S., Wong, A.S., Leung, P.C., 2007. Differential role of gonadotropin-releasing hormone on human ovarian epithelial cancer cell invasion. Endocrine 31, 311-320.

Drouillat, B., Hillery, A.M., Dekany, G., Falconer, R., Wright, K., Toth, I., 1998. Novel liposaccharide conjugates for drug and peptide delivery. J. Pharm. Sci. 87, 25-30.

Egleton, R., Davis, T., 2005. Development of neuropeptide drugs that cross the blood-brain barrier. Neurotherapeutics 2, 44-53.

Franklin, J., Hislop, J., Flynn, A., McArdle, C.A., 2003. Signalling and anti-proliferative effects mediated by gonadotrophin-releasing hormone receptors after expression in prostate cancer cells using recombinant adenovirus. J Endocrinol 176, 275-284.

Gibbons, W.A., Hughes, R.A., Charalambous, M., Christodoulou, M., Szeto, A., Aulabaugh, A.E., Mascagni, P., Toth, I., 1990. Lipidic peptides, I. Synthesis, resolution and structural elucidation of lipidic amino acids and their homo- and hetero-oligomers. Liebigs Ann. Chem. 1990, 1175-1183.

Griffin, B.D., CM O', 2011. Opportunities and challenges for oral delivery of hydrophobic versus hydrophilic peptide and protein-like drugs using lipid-based technologies. Therapeutic Delivery 2, 1633-1653.

Griffiths, E.C., Mcdermott, J.R., 1983. Enzymic Inactivation of Hypothalamic Regulatory Hormones. Molecular and cellular endocrinology 33, 1-25.

Grundker, C., Volker, P., Schulz, K.D., Emons, G., 2000. Luteinizing hormone-releasing hormone agonist triptorelin and antagonist cetrorelix inhibit EGF-induced c-fos expression in human gynecological cancers. Gynecologic oncology 78, 194-202.

Hollosy, F., Lorand, T., Orfi, L., Eros, D., Keri, G., Idei, M., 2002. 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 768, 361-368.

Johnsson, G., Regardh, C.G., 1976. Clinical pharmacokinetics of beta-adrenoreceptor blocking drugs. Clinical pharmacokinetics 1, 233-263.

Kang, S.K., Choi, K.C., Tai, C.J., Auersperg, N., Leung, P.C.K., 2001. 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 142, 580-588.

Karten, M.J., Rivier, J.E., 1986. Gonadotropin-Releasing-Hormone Analog Design - Structure-Function Studies toward the Development of Agonists and Antagonists - Rationale and Perspective. Endocr Rev 7, 44-66.

Koda, Y., Del Borgo, M., Wessling, S.T., Lazarus, L.H., Okada, Y., Toth, I., Blanchfield, J.T., 2008. Synthesis and in vitro evaluation of a library of modified endomorphin 1 peptides. Bioorganic and Medicinal Chemistry 16, 6286-6296.

Laimou, D.K., Katsara, M., Matsoukas, M.T.I., Apostolopoulos, V., Troganis, A.N., Tselios, T.V., 2010. Structural elucidation of Leuprolide and its analogues in solution: insight into their bioactive conformation. Amino acids 39, 1147-1160.

Leuschner, C., Enright, F.M., Gawronska-Kozak, B., Hansel, W., 2003. Human prostate cancer cells and xenografts are targeted and destroyed through luteinizing hormone releasing hormone receptors. The Prostate 56, 239-249.

Ling, N., Vale, W., 1975. Analogs of Luteinizing-Hormone Releasing Factor (Lrf) Synthesis and Biological-Activity of [(N-Alpha-Me)Leu7]Lrf and [D-Ala6,(N-Alpha-Me)Leu7]Lrf. Biochemical and biophysical research communications 63, 801-806.

Marelli, M.M., Moretti, R.M., Mai, S., Procacci, P., Limonta, P., 2007. 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. International journal of oncology 30, 261-271.

Mezo, G., Manea, M., 2010. Receptor-mediated tumor targeting based on peptide hormones. Expert Opinion on Drug Delivery 7, 79-96.

Mezo, G., Manea, M., Szabo, I., Vincze, B., Kovacs, M., 2008. New Derivatives of GnRH as Potential Anticancer Therapeutic Agents. Curr Med Chem 15, 2366-2379.

Millar, R.P., Lu, Z.L., Pawson, A.J., Flanagan, C.A., Morgan, K., Maudsley, S.R., 2004. Gonadotropin-releasing hormone receptors. Endocr Rev 25, 235-275.
Moradi, S.V., Mansfeld, F.M., Toth, I., 2013. Synthesis and in vitro evaluation of glycosyl derivatives of luteinizing hormone-releasing hormone (LHRH). Bioorganic and Medicinal Chemistry 21, 4259-4265.
Moradi, S.V., Varamini, P., Toth, I., 2014. The transport and efflux of glycosylated luteinising hormone-releasing hormone analogues in caco-2 cell model: contributions of glucose transporters and efflux systems. Journal of pharmaceutical sciences 103, 3217-3224.
Moradi, S.V., Varamini, P., Toth, I., 2015a. Evaluation of the Biological Properties and the Enzymatic Stability of Glycosylated Luteinizing Hormone-Releasing Hormone Analogs. The AAPS Journal 17, 1135-1143.
Moradi, S.V., Varamini, P., Toth, I., 2015b. Evaluation of the Biological Properties and the Enzymatic Stability of Glycosylated Luteinizing Hormone-Releasing Hormone Analogs. The AAPS journal.
Morgan, K., Stewart, A.J., Miller, N., Mullen, P., Muir, M., Dodds, M., Medda, F., Harrison, D., Langdon, S., Millar, R.P., 2008. Gonadotropin-releasing hormone receptor levels and cell context affect tumor cell responses to agonist in vitro and in vivo. Cancer Research 68, 6331-6340.
Nagy, A., Schally, A.V., 2005. Targeting of cytotoxic luteinizing hormone-releasing hormone analogs to breast, ovarian, endometrial, and prostate cancers. Biology of reproduction 73, 851-859.
Nomoto, M., Yamada, K., Haga, M., Hayashi, M., 1998. Improvement of intestinal absorption of peptide drugs by glycosylation: transport of tetrapeptide by the sodium ion-dependent D-glucose transporter. J. Pharm. Sci. 87, 326-332.
Nussey, S., Whitehead, S., 2001. Endocrinology: An Integrated Approach. BIOS Scientific Publishers, Oxford.
Pawson, A.J., Faccenda, E., Maudsley, S., Lu, Z.L., Naor, Z., Millar, R.P., 2008. Mammalian type I gonadotropin-releasing hormone receptors undergo slow, constitutive, agonist-independent internalization. Endocrinology 149, 1415-1422.
Pfleger, K.D., Bogerd, J., Millar, R.P., 2002. Conformational constraint of mammalian, chicken, and salmon GnRHs, but not GnRH II, enhances binding at mammalian and nonmammalian receptors: evidence for preconfiguration of GnRH II. Molecular Endocrinology 16, 2155-2162.
Ravenna, L., Salvatori, L., Morrone, S., Lubrano, C., Cardillo, M.R., Sciarra, F., Frati, L., Di Silverio, F., Petrangeli, E., 2000. Effects of triptorelin, a gonadotropin-releasing hormone agonist, on the human prostatic cell lines PC3 and LNCaP. J Androl 21, 549-557.
Ross, B.P., Falconer, R.A., Toth, I., 2008. N-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (N-Dde) Lipoamino Acids. Molbank 2, M566.
Schally, A.V., Redding, T.W., Comaruschally, A.M., 1984. Potential Use of Analogs of Luteinizing-Hormone-Releasing Hormones in the Treatment of Hormone-Sensitive Neoplasms. Cancer Treat Rep 68, 281-289.
Shiota, M., Tokuda, N., Kanou, T., Yamasaki, H., 2007. Incidence Rate of Injection-Site Granulomas Resulting from the Administration of Luteinizing Hormone-Releasing Hormone Analogues for the Treatment of Prostatic Cancer. Yonsei Medical Journal 48, 421-424.
Skinner, D.C., Albertson, A.J., Navratil, A., Smith, A., Mignot, M., Talbott, H., Scanlan-Blake, N., 2009. Effects of Gonadotrophin-Releasing Hormone Outside the Hypothalamic-Pituitary-Reproductive Axis. J Neuroendocrinol 21, 282-292.
Steyn, F.J., Wan, Y., Clarkson, J., Veldhuis, J.D., Herbison, A.E., Chen, C., 2013. Development of a Methodology for and Assessment of Pulsatile Luteinizing Hormone Secretion in Juvenile and Adult Male Mice. Endocrinology 154, 4939-4945.
Toth, I., 1994. A novel chemical approach to drug delivery: lipidic amino acid conjugates. J. Drug Target. 2, 217-239.
Turk, B.G., Dereli, T., Dereli, D., Akalin, T., 2007. Leuprolide acetate-induced leukocytoclastic vasculitis. Acta obstetricia et gynecologica Scandinavica 86, 892-893.
Varamini, P., Mansfeld, F.M., Blanchfield, J.T., Wyse, B.D., Smith, M.T., Toth, I., 2012a. Lipo-Endomorphin-1 Derivatives with Systemic Activity against Neuropathic Pain without Producing Constipation. PLoS One 7, e41909.
Varamini, P., Mansfeld, F.M., Blanchfield, J.T., Wyse, B.D., Smith, M.T., Toth, I., 2012b. Synthesis and Biological Evaluation of an Orally Active Glycosylated Endomorphin-1. J. Med. Chem. 55, 5859-5867.

FIGURES

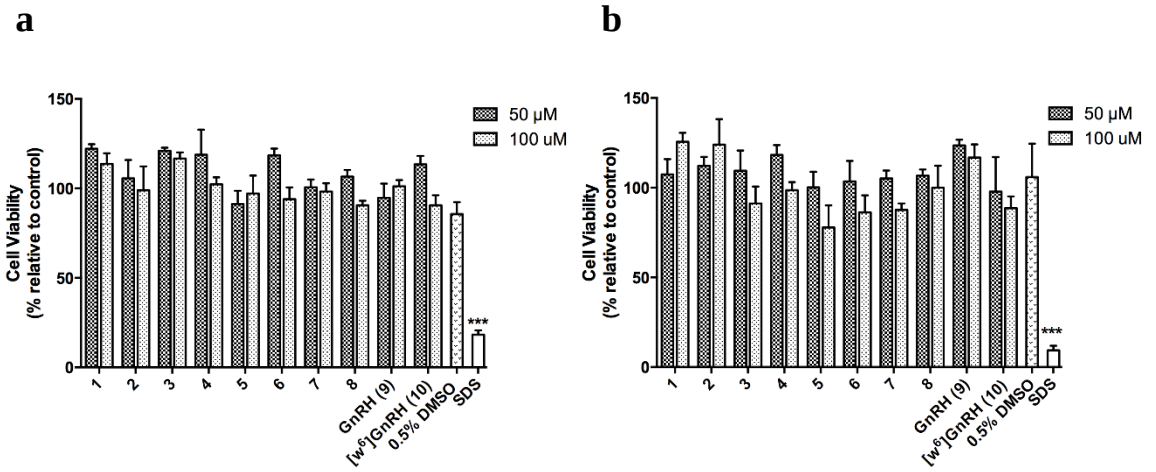


Figure 1. Toxicity of glycolipopeptides **1**-**8** in (a) PBMCs and (b) isolated rat pituitary cells, as a percentage of cells treated with PBS (mean ± StDev, performed in three independent experiments, each in triplicate) after 48 h incubation. Statistical analysis was performed using a one-way ANOVA followed by Dunnett's post-hoc test and comparison with the DMSO-negative control group.

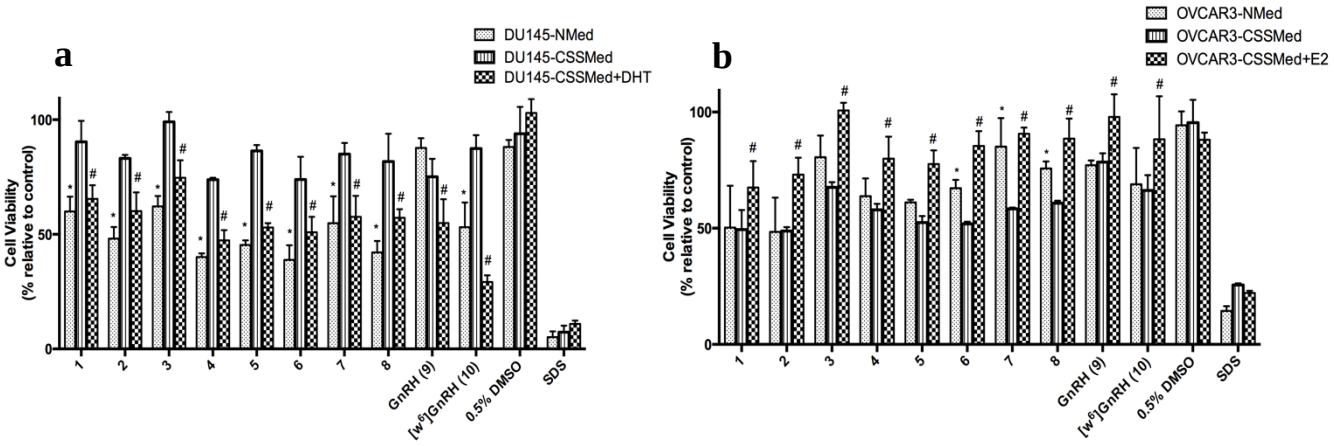


Figure 2. Effect of the reconstitution of the cell growth media with DHT and E2 on the sensitivity of (**a**) DU145 and (**b**) OVCAR-3 cells. Cells were grown in steroid free (CSS) media for 48 h then E2 (5 nM) or DHT (50 nM) was added to the media and incubated for 48 h. Treatment was commenced using glycolipid-modified GnRH analogs at 50 µM. * $p<0.05$ experiments performed in normal media vs. CSS media. # $p<0.05$ experiments performed in steroid reconstituted media vs. CSS media.

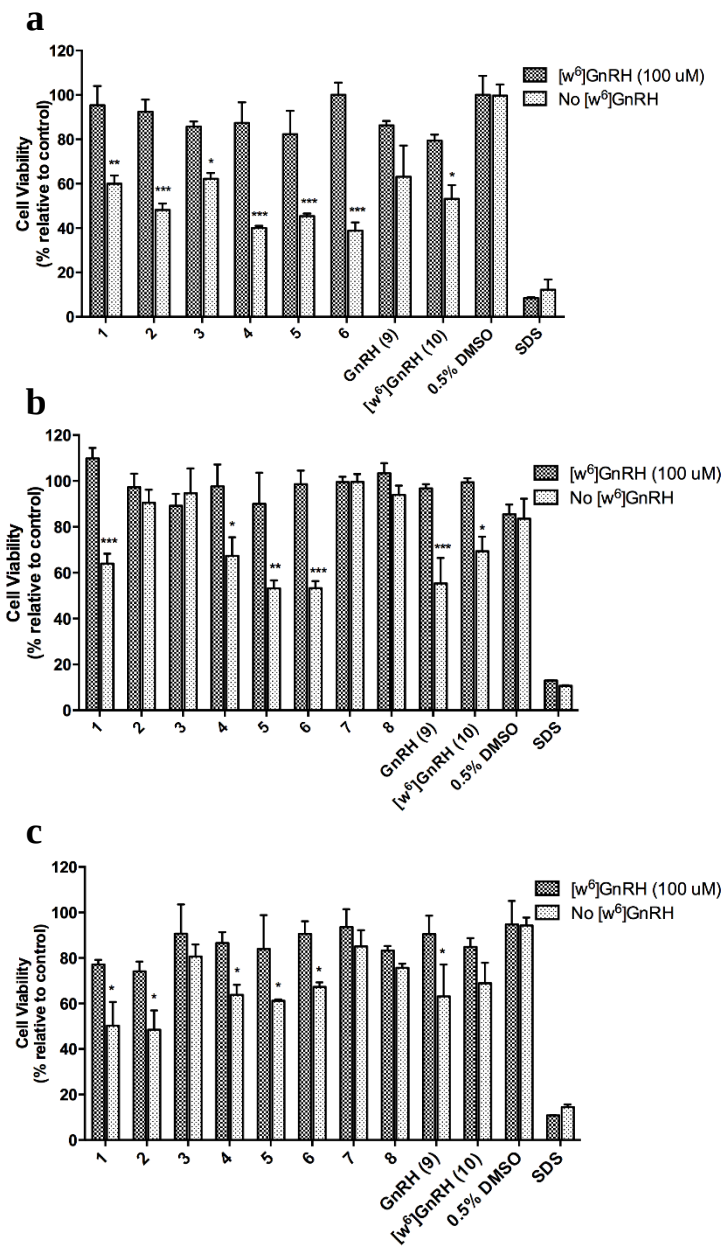


Figure 3. Receptor-mediated antiproliferation in (**a**) DU145, (**b**) LNCaP, and (**c**) OVCAR-3 cells. Cells were pretreated with 100 µM triptorelin for 2 h and then the supernatant was replaced with compounds **1-8** and control peptides in fresh media at 50 µM. MTT assay was performed after 48 h (mean ± StDev, performed in three independent experiments, each in triplicate). Statistical analysis was performed using a one-way ANOVA (* $p<0.05$; **, $p< 0.01$, *** $p< 0.001$, difference between pretreated and untreated groups).

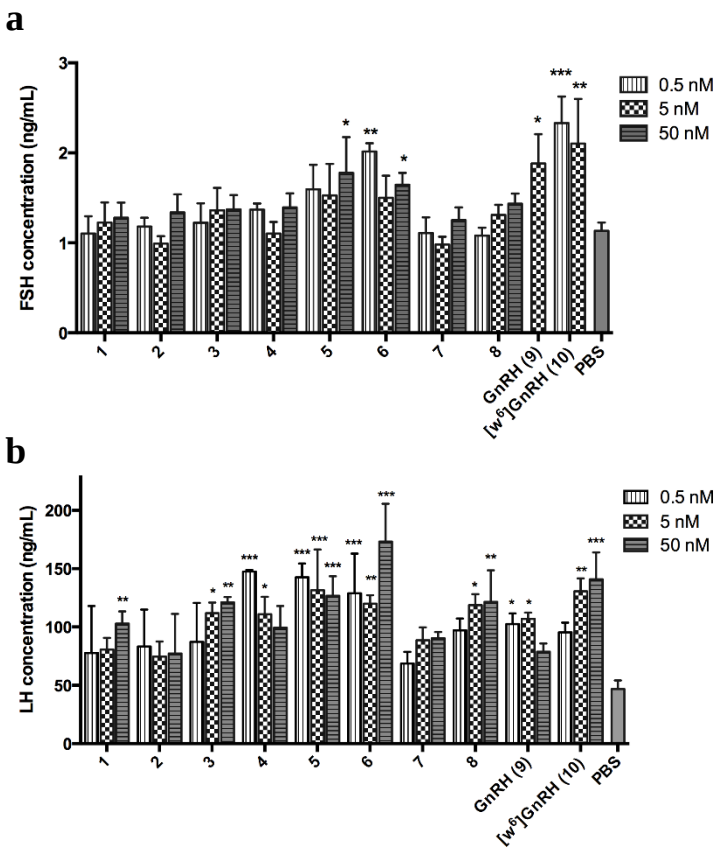


Figure 4. Effect of GnRH derivatives on the release of (**a**) FSH and (**b**) LH from rat pituitary cells. Statistical analysis was performed using a one-way ANOVA followed by the Dunnett's post hoc test (* p<0.05; **, p< 0.01, *** p< 0.001, increase in the FSH and LH level when compared to the PBS (negative control group).

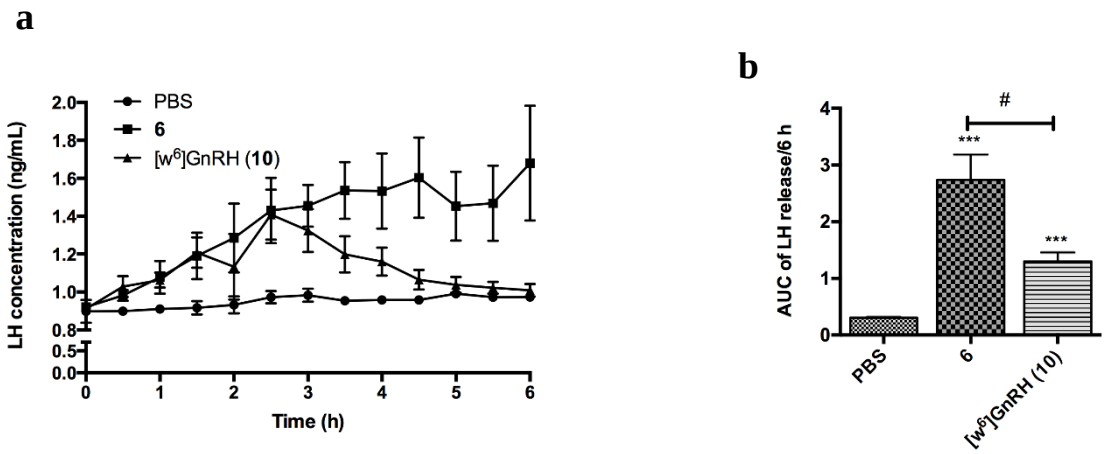
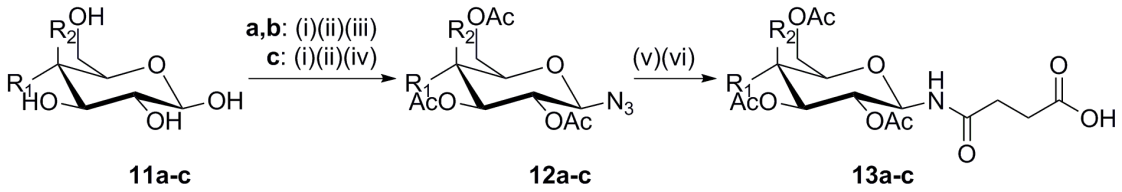


Figure 5. Efficacy of the lead compound **6** on the release of LH after subcutaneous administration in mice. (**a**) Plasma LH levels during the first 6 h post treatment and (**b**) the measured AUC over the 6 h sampling period following administration of glycolipopeptide **6**, triptorelin (or [w^6]GnRH, 50 µmol/mouse), or PBS (Mean ± StDev (n=5)). Statistical analysis was performed using a one-way ANOVA followed by the Dunnett's post hoc test. *** p<0.001 when compounds **6** and triptorelin were compared to PBS group (negative control) and # p<0.05 when compound **6** was compared with triptorelin.



Scheme 1. Reaction conditions. (i) $LiClO_4$, acetic anhydride, 40 °C, overnight; (ii) HBr (32%), AcOH, RT, 3 h; (iii) NaN_3, NBu_4HSO_4, $NaHCO_3$ (saturated), DCM, RT, overnight; (iv) NaN_3, acetone, water, RT, overnight; (v) DCM, H-cube, flow 2 mL/min, pH_2 40 bar, 35 °C, 1 h; (vi) succinic anhydride, pyridine, DMAP, DCM, 4 °C to RT, overnight.

Table 1. Chemical structures of the synthesized carbohydrate derivatives.

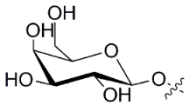
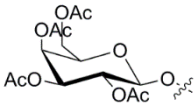
	Glucose		Galactose		Lactose	
	11a	**12a, 13a**	**11b**	**12b, 13b**	**11c**	**12c, 13c**
R_1	OH	OAc	H	H		
R_2	H	H	OH	OAc	H	H

Table 2. Composition of GnRH derivatives **1-8**.

Compound	**Amino acid sequence**											
1	Glc	C12	Gln	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly
2	Gal	C12	Gln	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly
3	Lac	C12	Gln	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly
4	Glc	C12	Gln	His	Trp	Ser	Tyr	D-Trp	Leu	Arg	Pro	Gly
5	Gal	C12	Gln	His	Trp	Ser	Tyr	D -Trp	Leu	Arg	Pro	Gly
6	Lac	C12	Gln	His	Trp	Ser	Tyr	D -Trp	Leu	Arg	Pro	Gly
7	-	Lac	Gln	His	Trp	Ser	Tyr	Gly	C12	Arg	Pro	Gly
8	-	Lac	Gln	His	Trp	Ser	Tyr	D -Trp	C12	Arg	Pro	Gly
9 (GnRH)	-	-	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly
10 ([w^6]GnRH /triptorelin)			pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly

Glc: glucose; Gal: galactose, Lac: lactose; C12: 2-amino-D,L-dodecanoic acid.

Table 3. Caco-2 cell homogenate stability ($t_{1/2}$) and Caco-2 cell membrane permeability (P_{app}) of GnRH glycolipids (**1-8**) and controls (**9, 10**).

Compound	$t_{1/2}$ **(min, Caco-2)**	P_{app} **(×10^{-7}, cm/s)** [a]
1	42.1 ± 4.1	8.5 ± 3.97
2	45.5 ± 3.1	1.4 ± 0.8
3	61.3 ± 5.2 *	32.8 ± 2.4*
4	109.7 ± 6.5	6.2 ± 0.33
5	106.5 ± 4.8*	8.8 ± 1.6
6	124.1 ± 4.1*	19.2 ± 1.1*
GnRH **(9)**	10.2 ± 4.1	1.2 ± 0.31
[w^6]GnRH **(10)**	19.8 ± 2.8	2.8 ± 0.50
Propranolol	N/A	75.6 ± 15*
Mannitol	N/A	0.8 ± 0.2
7	39.8 ± 3.0 *	6.8 ± 3.5[b]
8	41.6 ± 3.2	8.9 ± 0.63
GnRH **(9)**	13.3 ± 3.7	2.2 ± 0.94
[w^6]GnRH **(10)**	21.8 ± 1.9	9.9 ± 2.5
Propranolol	N/A	134 ± 8.1*
Mannitol	N/A	1.4 ± 0.03

[a]The apparent permeability (P_{app}, cm/s) through Caco-2 cell monolayers. The data were quantified by LC/MS and each point is expressed as mean ± StDev (n = 4 from three independent experiments). Statistical analysis was performed using a one-way ANOVA followed by Dunnett's post-hoc test and values compared with GnRH (* $p < 0.05$).
[b]The values for compounds 7 and 8 were obtained from a different experiment for both stability and membrane permeability along with their corresponding controls (GnRH and [w6]GnRH).

Table 4. Antiproliferative activity of GnRH glycolipids in different cell lines.

GnRH Glycolipids	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	GnRH (**9**)	[w^6]GnRH (**10**)
DU145 IC_{50} (μM)	57.4 ± 3.1	61.1 ± 2.9	49.3 ± 2.3	39.4 ± 0.9*	37.4 ± 1.2*	36.4 ± 3.7*	61.2 ± 6.7	62.1 ± 2.8	>100	62.1 ± 6.2
LNCap IC_{50} (μM)	83.1 ± 4.3	>100	>100	69.3 ± 8.1	57.6 ± 3.4*	52.2 ± 3.1*	>100	>100	>100	73.4 ± 6.3
PC3 IC_{50} (μM)	>100	>100	>100	91.2 ± 8.8	83.5 ± 7.5	89.7 ± 9.1	>100	92.4 ± 8.2	>100	98.1 ± 6.4
OVCAR-3 IC_{50} (μM)	41.8 ± 3.2*	46.3 ± 2.8*	>100	63.2 ± 5.5	65.6 ± 5.6	59.9 ± 7.1	>100	88.5 ± 6.1	90.1 ± 3.4	67.7 ± 4.4
SKOV-3 IC_{50} (μM)	81.2 ± 5.3	88.4 ± 4.9	>100	>100	>100	>100	>100	>100	>100	>100

The IC_{50} values (μM) were estimated from concentration-response curves using nonlinear regression for inhibition of cell growth. Data are expressed as mean ± SD from at least three independent experiments, each in triplicate. Statistical analysis was performed using a two-way ANOVA (* $p < 0.05$, the IC_{50} for each compared with that of their corresponding parent peptide for the same cell line).