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RESEARCH PAPER

Stability, Permeability and Growth-Inhibitory Properties of Gonadotropin-Releasing Hormone Liposaccharides

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ABSTRACT

Purpose In this study we aimed to address the poor drug-like properties of Gonadotropin-Releasing Hormone (GnRH) peptide through modification with lipids and carbohydrates.

Methods GnRH peptide was conjugated to 2-amino-D,L-octanoic acid (C8) and 2-amino-D,L-dodecanoic acid (C12) in monomer and dimer, along with (**6**–**9**) or without (**2**–**5** and **11**) a glucose moiety. Peptides were tested for their biological activity using different tumour cell lines. The toxicity of the constructs was evaluated in peripheral blood mononuclear cells (PBMC).

Results All (glyco)lipopeptides showed improved metabolic stability in Caco-2 cell homogenates. Those with single lipid moiety (**2**, **4** and **8**) exhibited prodrug-like properties. Permeability across Caco-2 cell monolayers was enhanced in the dimer C8-modified (glyco)lipopeptide (**3**) and the lipopeptide with C12 inserted mid-sequence (**11**). Most of the constructs showed moderate-to-high antiproliferative activity against GnRH-receptor positive DU145 and OVCAR-3 cells (up to 60%). Compound **11** was the most effective with $IC_{50} = 26.4 \pm 1.07\ \mu g.ml^{-1}$, which was comparable to triptorelin ($25.1 \pm 1.14\ \mu g.mL^{-1}$). The sensitivity of OVCAR-3 cells to the effect of all analogues except for **11** decreased significantly in estrogen-reconstituted media. Only compounds **2**, **4**, **5** and **8** showed a steroid-dependent effect in DU145 cells. No compounds exhibited significant toxicity on PBMCs.

Conclusion These results indicated lipidation and glycosylation improves the druggability of GnRH and could lead to an increased direct antitumour activity in some hormone dependent and independent reproductive cancers.

KEY WORDS antiproliferative activity · Gonadotropin-Releasing Hormone · lipoamino acid · liposaccharide · peptide delivery

ABBREVIATIONS

°C	Degrees Celsius
Ac2O	Acetic anhydride
AcOH	Acetic acid
ANOVA	Analysis of variance
AUC	Area under the curve
BBB	Blood brain barrier
Boc	*tert*-Butyloxycarbonyl
BSA	Bovine serum albumin
DCM	Dichloromethane
DIPEA	N,N-Diisopropylethylamine
DIPEA	N,N-Diisopropylethylamine
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionisation
EtOAc	Ethyl acetate
EtOH	Ethanol
FBS	Fetal bovine serum
Fmoc	9-Fluorenylmethoxycarbonyl
FSH	Follicle-stimulating hormone
Glc	Glucose
GLUT-1	Glucose transporter 1
GnRH	Gonadotropin-Releasing Hormone
HATU	O-(7-Azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate
HBSS	Hank's buffered salt solution
HBTU	O-Benzotriazole-N,N,N′,N′-tetramethyluronium-hexafluorophosphate
HCl	Hydrochloric acid
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	Hydrogen fluoride
HPLC	High-performance liquid chromatography
Hz	Hertz

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LC-MS	Liquid Chromatography-Mass Spectrometry
LH	Luteinising hormone
LHRH	Luteinising hormone-releasing hormone
LNCaP	Prostate adenocarcinoma cells from left supraclavicular lymph node
m/z	Mass-to-charge ratio
MBHA	4-Methylbenzhydrylamine
MeCN	Acetonitrile
MeOH	Methanol
MS	Mass spectrometry
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MW	Molecular weight
ND	Not determined
NHS	*N*-hydroxysuccinimide
NMR	Nuclear magnetic reasonance
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
RP-HPLC	Reverse-phase-high performance liquid chromatography
Rt	Retention time
t1/2	Half-life
TEER	Transepithelial electrical resistance
TFA	Trifluoroacetic acid
UV	Ultraviolet
δ	Chemical shift in parts per million downfield from tetramethylsilane

AMINO ACIDS

Arg R	Arginine
Glu E	Glutamic acid
Gly G	Glycine
His H	Histidine
Leu L	Leucine
Pro P	Proline
Ser S	Serine
Trp W	Tryptophan
Tyr Y	Tyrosine

INTRODUCTION

Gonadotropin-Releasing Hormone (GnRH, *p*-EHWSYGLRPG) is an endogenous decapeptide released from the hypothalamus. It plays a critical role in regulating the functions of reproductive organs by controlling the levels of gonadotropins, luteinising hormone (LH) and follicle-stimulating hormone (FSH). These hormones, in turn, encourage the secretion of sex steroids, namely testosterone, estrogen and progesterone. Sustained delivery of GnRH ultimately down-regulates GnRH receptors (GnRH-R), which results in a significant reduction in endogenous sex hormones.

Before the 1940s, surgical castration was a standard practice as a treatment for men with prostatic cancers. In 1941, Huggins and Hodges suggested a pharmaceutical alternative to surgery, mimicking the actions of castration with the administration of diethylstilbestrol (1). More recently, a growing number of agonists and antagonists have been developed based on the primary structure of GnRH. These compounds represent nearly a quarter of the total peptide drug market, which was estimated to be valued at US$11 billion in 2011 (2). After sequencing and characterising GnRH in the 1970s, a number of GnRH-R agonists, such as buserelin, goserelin, triptorelin, and leuprolide were discovered and marketed (3). Leuprolide alone has reached sales over US$2 billion worldwide (4). The administration of a GnRH agonist in conjunction with an antiandrogen became standard treatment for androgen-dependant cancers, and was an acceptable alternative to surgical castration (5).

Apart from their role in inhibiting pituitary gonadotropins, GnRH-mediated pathways have been proven to potentiate or deteriorate the growth of certain types of cancer cells, such as breast, endometrial, ovarian and prostate cells (6). The growth inhibitory effect of GnRH analogues in postmenopausal women with breast cancer suggests direct antiproliferative effects that are independent of their actions in decreasing sex steroid hormones (7). It has become increasingly clear that GnRH-R are expressed in cancer tissues related (*e.g.* prostate, ovarian) (8) or unrelated (*e.g.* brain, lung, liver) to the reproductive system (9). Activation of tumour GnRH-R by its agonists has elicited strong antiproliferative, antimetastatic, and antiangiogenic activity (10). A large number of studies have been performed on different tumour cell lines demonstrating a direct GnRH-R-mediated cell growth inhibition. This effect has been shown to be directly dependent on the level of GnRH-R expression in the particular cell line (11).

Current GnRH-based therapeutics require regular injections or intramuscular drug delivery systems for controlled release. Although the parenteral route is the most common delivery of GnRH, oral delivery is the preferred route of administration with high levels of patient acceptance and long-term compliance. Comprehensive research in addressing the challenges associated with the oral delivery of peptides has led to novel delivery systems including their formulation into nanoparticles, conjugation to large carrier molecules, and use of covalently bound lipid moieties (12). Increasing the lipophilicity of unfavourably hydrophilic compounds was used to improve absorption by passive diffusion across epithelial barriers, such as the gastrointestinal tract and central nervous system (13). Lipophilic moieties coupled to peptides conferred the characteristics essential for intestinal delivery. They were also capable of protecting a usually labile peptide from enzymatic degradation (14). These advantages could considerably enhance the bioavailability of peptides. Modifications such as the addition of lipidic moieties or cyclisation have been

attempted to not only increase stability but also to allow transport across biological systems (15). 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 (16). The lipidic nature of lipopeptide conjugates contributes to membrane-like characteristics with all the benefits of increased lipophilicity (16). Evidence has shown that lipoamino acids can be released from their parent peptides *in vitro* (17) and thus can be used for development of lipopeptide-based prodrugs.

Increasing the lipophilicity of compounds, although mainly beneficial, may also decrease their solubility in water, affecting their distribution in formulations and also in the body. In the delivery of bioactive compounds, a balance between lipophilic and hydrophilic characters must be considered, and liposaccharide-based delivery systems can be used to address this problem (13). Glycosylation of peptides may also confer many other advantages, including enhanced physicochemical properties, stability, permeability and targeting (18). The use of both lipids and carbohydrates in one delivery system affords the benefits of both approaches by enhancing the bioavailability of peptides (13). It is proposed that conjugation of the GnRH peptide to lipophilic and glycosyl moieties results in an increased stability and membrane permeability of the peptide, which can contribute to higher efficacy as reported earlier for other GnRH analogues (19).

In this study, a series of GnRH (glyco)lipopeptides were successfully synthesised and evaluated *in vitro* for their stability and permeability in Caco-2 cells. Furthermore, different tumour cell lines with various GnRH-R expression levels were used to investigate the effect of the GnRH (glyco)lipopeptides on prostate and ovarian cancer cell proliferation.

EXPERIMENTAL SECTION

Materials and Methods

Protected L-amino acids and resins were purchased from Novabiochem (Läufelfingen, Switzerland), Reanal (Budapest, Hungary) and Mimotopes (Melbourne, Australia). Peptide synthesis grade DMF was supplied by Labscan (Dublin, Ireland) and filtered through a 0.45 μM (47 mm) nylon filter before each use. TFA and DIPEA were supplied by Merck KGaA (Darmstadt, Germany), HATU and HBTU from Mimotopes (Melbourne, Australia). Analytical grade MeCN was purchased from Scharlau (Barcelona, Spain) or Labscan (Dublin, Ireland). All other reagents were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) at the highest available purity. Biological reagents were mainly purchased from Gibco-BRL (Grand Island, NY), with the exception of HBSS, Drabkin's reagent and Brij1 35 detergent (Sigma-Aldrich, Castle Hill, NSW, Australia), ^{14}C-mannitol (Amersham Biosciences, Piscataway, NJ), and propanolol (Sigma-Aldrich, Castle Hill, NSW, Australia). Tissue culture flasks (TPP1 75 cm^2) and 96-well plates were purchased from Becton Dickinson; Transwell polycarbonate inserts (mean pore size ¼ 0.45 lm, 6.5 mm diameter) were supplied by Costar (Cambridge, MA). Caco-2 cells were obtained from the American type culture collection (Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and 1% nonessential amino acids in an atmosphere of 95% humidity and 5% CO_2 at 37°C. The medium was changed every second day. After reaching ~80% confluence, the cells were subcultured using PBS and 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and seeded in tissue-culture plates. Charcoal Stripped Fetal Bovine Serum was resourced from Life Technologies (Gibco).

RP-HPLC analysis was carried out using a Shimadzu (Kyoto, Japan) instrument, with Grace Vydac C4, C8, or C18 (Columbia, Maryland, USA) Protein/Peptide columns (250×3×4.6 mm, 5 μm). Analysis was achieved using a gradient of solvent A [H_2O + 0.1% TFA] and solvent B [MeCN/H_2O (90:10) + 0.1% TFA]. A gradient of 0–100% B over 30 min at 1 ml.min^{-1} was employed and absorbance detected at 214 nm. Crude peptides were purified using a preparative Waters (Milford, Massachusetts, USA) Delta Prep 600 system or a Shimadzu (LC-20AT, SIL-10A, CBM-20A, SPD-20AV, FRC-10A) instrument with a Grace Vydac C18 column (250×3×22 mm, 10 μm). Separation was achieved using a gradient of 0–100% B at a flow rate of 10–20 ml.min^{-1}, and absorbance detected at 230 nm.

^{1}H-NMR spectra were recorded on a Bruker ADVANCE spectrometer at 300 MHz in $CDCl_3$. Electrospray ionisation-mass spectrometry (ESI-MS) was performed on a triple quadrupole Perkin-Elmer-Sciex API3000 mass spectrometer using a solvent mixture of A [H_2O + 0.1% AcOH] and B [MeCN/H2O + 0.1% AcOH]. LC-MS analyses were run at 0.5 ml.min^{-1} in gradient mode (0–100% B) on a Phenomenex Luna C18 column (2.1×3×50 mm, 5 μm) equipped with a C18 pre-column. Data were acquired and analysed using Analyst 1.4 (Applied Biosystems/MDS Sciex, Toronto, Canada) software. A 1:10 splitter was used at the entrance of the mass spectrometer source. Results were quantified based upon ≥8 point standard curves (0.05–200 μM).

Chemical Synthesis

Lipidic Moieties

Racemic 2-aminooctanoic and 2-aminododecanoic acids (C8- and C12-lipoamino acids) were synthesised according to Gibbons *et al.* using the appropriate 1-bromoalkanes and diethyl acetoamidomalonate (20). The formed C8- and C12-lipoamino acids were then N$^{\alpha}$-Boc protected.

2-*tert*-Butoxycarbonylaminooctanoic acid. (data corresponds to that reported in the literature, (13)) 1.57 g, 63.0%; R_f=0.58 (DCM/MeCN/AcOH); ESI-MS ($C_{13}H_{25}NO_4$, 259.2): m/z=282.2 $[M + Na]^+$ (calcd. 282.2), 304.2 $[M + 2Na–GnRH]^+$ (calcd. 304.2); ^1H NMR, ($CDCl_3$): δ=4.96-4.93 (1H, d, OCONH), 4.26 (1H, m, α-CH), 1.83-1.67 (2H, m, β-CH), 1.50-1.42 (9H, m, $C(CH_3)_3$), 1.36-1.20 (8H, m, 4 CH_2), 0.86 (3H, t, CH_3).

2-*tert*-Butoxycarbonylaminododecanoic acid. (data corresponds to that reported in the literature, (21)) 1.31 g, 54.0%; R_f=0.65 (DCM/MeCN/AcOH); ESI-MS ($C_{17}H_{33}NO_4$, 315.2): m/z=316.4 $[M + GnRH]^+$ (calcd. 316.2), 338.2 $[M + Na]^+$ (calcd. 338.2); ^1H NMR, ($CDCl_3$): δ=4.94-4.92 (1H, d, OCONH), 4.26 (1H, m, α-CH), 1.82-1.67 (2H, m, β-CH), 1.42 (9H, m, $C(CH_3)_3$), 1.23 (16H, m, 8 CH_2), 0.86 (3H, t, CH_3).

2-*tert*-Butoxycarbonylaminododecanoic acid *N*-hydroxysuccininyl ester, Boc-C_{12}-NHS. 2-*tert*-Butoxycarbonylaminododecanoic acid (260 mg, 0.82 mmol) and *N*-hydroxysuccinimide (105 mg, 91 mmol) were added to dry EtOAc (10 ml). Dicyclohexylcarbodiimide (188 mg, 91 mmol) in dry EtOAc (5 ml) was added and the reaction was stirred overnight at RT. The mixture was filtered to remove dicyclohexylurea and the EtOAc was removed *in vacuo* to yield the crude product as white crystals. The solid was recrystallised from EtOH to yield the pure 2-*tert*-butoxycarbonylaminododecanoic acid *N*-hydroxysuccininyl ester - 312 mg, 92%; R_f ($CHCl_3$:MeOH, 10:0.7)=0.95; ESI-MS ($C_{21}H_{36}N_2O_6$, 412.3): *m/z*=430.4 $[M + H_2O]^+$ (calcd. 430.3), 435.4 $[M + Na]^+$ (calcd. 435.3), 451.3 $[M + K]^+$ (calcd. 451.3), 825.8 $[2 M + GnRH]^+$ (calcd. 825.6), 842.7 $[2 M + H_2O]^+$ (calcd. 841.6), 847.7 $[2 M + Na]^+$ (calcd. 847.6); ^1H NMR ($CDCl_3$) δ=4.94-4.92 (1H, d, OCONH), 4.26 (1H, m, α-CH), 2.84 (4H, s, OCCH), 2.07-1.71 (2H, m, β-CH), 1.46 (9H, m, $C(CH_3)_3$), 1.26 (16H, m, 8 CH_2), 0.88 (3H, t, CH_3).

Peptide Synthesis

GnRH Peptides (1–11). Peptides were synthesised using standard manual solid-phase peptide synthesis protocols and then purified. Synthesis occurred on *p*-4-methyl benzhydryl amine (substitution 0.45 $mmol.g^{-1}$) resin, which was swollen in DMF/DIPEA for approximately 1 h. Synthesis was generally carried out using 4 equiv. Boc-L-amino acids. The peptides were then coupled using amino acids activated for 5 min with an equimolar amount of 0.5 M HBTU in DMF and 6 equiv. DIPEA, then mixed with the resin for 30–45 min. The N-terminal *p*Glu residue was substituted with Glu in peptides **1**–**9**. The protecting groups used were Boc groups for α-amino-termini, tosyl for Arg, 2,4-dinitrophenol for His, benzyl for Ser, formyl for Trp, 2-bromobenzyloxycarbonyl for Tyr and cyclohexyl for Glu. The Boc protecting groups were removed from the amino acids using neat TFA.

Prior to final Boc deprotection, the formyl and DNP protecting groups were removed with 20% piperidine in DMF and 20% 2-mercaptoethanol/10% DIPEA in DMF, respectively. After each manipulation the resin was washed with DMF. After the last coupling, all terminal Boc groups were removed with neat TFA and the resin thoroughly washed with DMF. The resin was then washed with DCM and MeOH and left to dry *in vacuo* overnight.

The resin was cleaved from the peptides using anhydrous HF at a concentration of 10 $ml.g^{-1}$ of resin and 5% *p*-cresol at −5°C for 1–2 h (22). Following this, the peptide was precipitated in diethyl ether, washed through a polyethylene frit, then dissolved in 1:1 MeCN:H_2O and lyophilised. The remaining amorphous powder was collected and weighed.

[E]GnRH, **1**: purified yield=5.0 mg, 25%; HPLC (0–100% solv. B [MeCN/H_2O (90:10) + 0.1% TFA], 30 min): t_R (column) (C18)=11.89 min; t_R (C4)=12.37 min; ESI-MS ($C_{55}H_{77}N_{17}O_{14}$, 1199.6) m/z: 400.7 $[M + 3H]^{3+}$ (calcd. 400.9), 600.8 $[M + 2H]^{2+}$ (calcd. 600.8).

GnRH, **10**: purified yield=4.5 mg, 23%; HPLC (0–100% solv. B, 30 min): t_R (column) (C18)=13.43; ESI-MS ($C_{55}H_{75}N_{17}O_{13}$, 1181.6) m/z: 591.8 $[M + 2H]^{2+}$ (calcd. 591.8).

2: purified yield=5.0 mg 25%; HPLC (0–100% solv. B, 30 min): t_R (column) (C18)=13.67, 14.10; t_R (C4)=14.20, 14.60; ESI-MS ($C_{63}H_{92}N_{18}O_{15}$, 1340.7) m/z: 447.9 $[M + 3H]^{3+}$ (calcd. 447.9), 671.3 $[M + 2H]^{2+}$ (calcd. 671.4).

3: purified yield=5.4 mg, 27%; HPLC (0–100% solv. B, 30 min): t_R (column) (C18)=15.69, 16.44, 17.23, 17.40; t_R (C4)=16.13, 16.84, 17.57, 17.70; ESI-MS ($C_{71}H_{107}N_{19}O_{16}$, 1481.8) m/z: 494.9 $[M + 3H]^{3+}$ (calcd. 494.9), 741.9 $[M + 2H]^{2+}$ (calcd. 741.9).

4: purified yield=5.2 mg, 26%; HPLC (0–100% solv. B, 30 min): t_R (column) (C18)=16.42, 17.16; t_R (C4)=16.72, 17.34; ESI-MS ($C_{67}H_{100}N_{18}O_{15}$, 1396.8) m/z: 466.6 $[M + 3H]^{3+}$ (calcd. 466.6), 699.4 $[M + 2H]^{2+}$ (calcd. 699.4).

5: purified yield=5.4 mg, 27%; HPLC (0–100% solv. B, 30 min): t_R (column) (C18)=12.99, 21.75, 22.53; t_R (C4)=20.51, 21.12, 21.58; ESI-MS ($C_{79}H_{123}N_{19}O_{16}$, 1593.9) m/z: 798.8 $[M + 2H]^{2+}$ (calcd. 798.0).

6: purified yield=9.8 mg, 49%; HPLC (0–100% solv. B, 30 min): t_R (column) (C18)=16.47; t_R (C4)=13.45, 13.70; ESI-MS ($C_{73}H_{107}N_{19}O_{22}$, 1601.8) m/z: 802.5 $[M + 2H]^{2+}$ (calcd. 801.9), 1603.1 $[M + GnRH]^+$ (calcd. 1602.8).

7: purified yield = 6.4 mg, 32%; HPLC (0–100% solv. B, 30 min): t_R (column) (C18) = 19.10, 19.31, 19.35, 19.75; t_R (C4) = 15.01, 16.40, 16.72; ESI-MS ($C_{81}H_{122}N_{20}O_{23}$, 1742.9) m/z: 873.0 $[M + 2H]^{2+}$ (calcd. 872.5), 1744.2 $[M + GnRH]^{+}$ (calcd. 1743.9).
8: purified yield = 4.6 mg, 23%; HPLC (0–100% solv. B, 30 min): t_R (column) (C18) = 19.16, 19.80; t_R (C4) = 15.48, 16.28; ESI-MS ($C_{77}H_{115}N_{19}O_{22}$, 1657.9) m/z: 830.2 $[M + 2H]^{2+}$ (calcd. 830.0), 1659.2 $[M + GnRH]^{+}$ (calcd. 1658.9).
9: purified yield = 3 mg, 15%; HPLC (0–100% solv. B, 30 min): t_R (column) (C18) = 24.2, 24.5; t_R (C4) = 20.25; ESI-MS ($C_{89}H_{138}N_{20}O_{23}$, 1855.0) m/z: 928.9 $[M + 2H]^{2+}$ (calcd. 928.5), 1857.3 $[M + GnRH]^{+}$ (calcd. 1856.0).

*Ester-Linked Lipoamino Acid-GnRH (**11**).* Using Fmoc chemistry, the peptide was assembled on Rink Amide MBHA resin (100–200 mesh, 0.59 mmol.g^{-1}, Peptides International, USA) using Fmoc-protected amino acids (4 equiv.), HATU (4 equiv.) and DIPEA (5 equiv.) activation. Synthesis was carried out using the following amino acids: Fmoc-Gly, Fmoc-Pro, Fmoc-Arg(Pbf), Fmoc-Leu, Fmoc-Tyr, Fmoc-Ser(tBu), Fmoc-Trp(Boc), Fmoc-His(Trt) and pyroglutamic acid. Each amino acid was double coupled and each coupling was carried out for 20 min. Fmoc protecting groups were removed using 20% piperidine/DMF (2 × 15 min). After each manipulation, the resin was washed with DMF. Esterification was achieved by first mixing the resin with 0.17 M triethylamine/DMF containing 4 Å molecular sieves under N_2. Boc-C12-NHS (4 equiv.) in DMF was then added and the reaction was mixed overnight at RT. The resin was then washed consecutively with DMF, DCM and MeOH and left to dry *in vacuo* overnight. Cleavage of the peptide was achieved using TFA/trisisopropylsilane/H_2O (95:2.5:2.5) for 1.5 GnRH. The crude peptide was then precipitated by the addition of cold diethyl ether, centrifuged to form a pellet, then dissolved in MeCN/H_2O/TFA (50:50:0.1) and lyophilised.

11: purified yield = 3.2 mg, 20%; HPLC (0–100% solv. B, 30 min): t_R (column) (C8) = 19.07; t_R (C4) = 16.27; ESI-MS ($C_{67}H_{98}N_{18}O_{14}$, 1378.8) m/z: 690.7 $[M + 2H]^{2+}$ (calcd. 690.4), 1380.2 $[M + GnRH]^{+}$ (calcd. 1379.8).

All crude peptides were purified using RP-HPLC on Vydac® C18 column (10 µm, 22 × 250 mm), with a gradient of solvent B (solvent A 0.1% TFA in H_2O; solvent B 90% acetonitrile and 0.1% TFA in H_2O) over 30 min with a flow rate of 10–20 ml.min^{-1}. The quality and purity of the peptide in the fractions was determined using ESI-MS and analytical RP-HPLC using a Vydac® C4, C8 or C18 column (5 µm 4.6 mm × 250 mm) at a flow rate of 1 ml.min^{-1} and a gradient of 0–100%, and/or 30–80% solvent B. Fractions containing pure compound were pooled and lyophilised overnight.

12 ([w^6]GnRH or triptorelin): purified yield = 22 mg, 63%; HPLC (0–100% solv. B, 30 min): t_R (column) (C18) = 18.23; ESI-MS ($C_{64}H_{82}N_{18}O_{13}$, 1311.3) m/z: 656.7 $[M + 2H]^{2+}$ (calcd. 656.7).

In vitro Experiments

Preparation of Stock Solutions

For each compound (including ^{14}C-mannitol), 200 µM solutions were prepared in HBSS buffer containing 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and adjusted to pH = 7.4 (HCl or NaOH). The peptides were initially dissolved in small amounts of DMSO, then diluted with HBSS buffer to give final 200 µM solutions with a DMSO concentration of 5–10% (*v/v*). Solutions were stored at −20°C.

Stability Assays

Caco-2 cells were seeded into a 75 cm^2 flask and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% nonessential amino acids, 100 U.ml^{-1} penicillin, and 100 µg.ml^{-1} streptomycin (Pen-Strep). The medium was changed every 2 days for 1–2 weeks, after which cells were washed sequentially with 0.02% EDTA and HBSS buffer solution, and sonicated with electric pulses produced by a Sonics VibraCell ultrasonic processor (Sonic and Materials Inc., Danbury, CN) to lyse the membranes. The cell samples were then centrifuged at 5°C and the protein content of the enzymatic extract determined using a Bio-Rad Protein Assay kit using albumin to produce a standard curve. The collected supernatant (0.8–0.9 mg.ml^{-1} protein) was aliquoted into individual wells of 96-well plates (100 µL/well). For each compound, four wells of a 96-well flat bottom plate were filled with 100 µL of 200 µM stock solution and the plates were then placed on an incubator shaker (Heidolph Inkubator & Titramax 1000, Schwabach, Germany) set to 400 rpm at 37°C. Samples (10 µL/well) of the reaction medium were taken at 5, 10, 15, 20, 30, 40, 50, 60 and 120 min, and added to 90 µL H_2O/TFA (5%) to stop enzymatic reactions. Samples were then analysed by LC-MS on a C18 column, using a ≥8 point standard curve (0.05–200 µM) for quantification.

Permeability Assays

Caco-2 cells (passage 45) were seeded at approximately 10^6 cells onto polycarbonate Transwell inserts and cultivated in DMEM supplemented with 10% FBS, 2 mM L-glutamine,

1% nonessential amino acids, and Pen-Strep. Cells were allowed to grow and differentiate for 21–26 days with the medium changed every second day. When confluent, cell monolayers were washed three times with HBSS buffer containing 25 mM HEPES (pH=7.4) and the integrity of the monolayers was assessed prior to and after each experiment by measuring TEER values using a Millicell-ERS system (Millipore Corp., Bedford, MA). For each compound, 100 µL of the 200 µM peptide or ^{14}C-mannitol solution was added to the apical compartment and 600 µL of HBSS buffer was added to the receiver compartment. Each compound was tested in quadruplicate. Plates were placed on an incubator shaker at 400 rpm and 37°C. At 30, 90, 120 and 150 min, 400 µL was sampled from the receiver compartment of each well and replaced with an equal amount of fresh HBSS buffer.

Collected samples containing ^{14}C-mannitol were diluted with 4 ml of Wallac OptiPhase HiSafe 3 liquid scintillation cocktail and their radioactivity measured using a Tri-Carb 2700 TR liquid scintillation spectrometer. Lipidic peptides were analysed by LC-MS on a C18 column, using a ≥8 point standard curve for quantification. Apparent permeability coefficients (*P*app, cm.s^{-1}) were determined using the following equation (23):

$$P_{\mathrm{app}} = \frac{\mathrm{d}C}{\mathrm{d}t}.\frac{V_{\mathrm{r}}}{A.C_0}$$

Where, d*C*/d*t* is the steady-state rate of change of the compound in the receiver chamber (mM, or dpm.ml^{-1}), *Vr* is the volume in the receiver chamber (ml), A is the surface area of the cell monolayer (cm^2) and C_0 is the initial concentration in the donor chamber (mM, or dpm.ml^{-1}).

Tumour Cell Proliferation Assay (MTT Assay)

A combination of steroid hormone-dependent and independent, sex-specific human carcinoma cell lines were used. The LNCaP (androgen-sensitive prostate adenocarcinoma), DU145 (androgen-independent human carcinoma) and OVCAR-3 (steroid hormone-sensitive ovarian carcinoma) human cell lines were also used and were kindly provided by Professor Judith Clements at the Translational Research Institute, Queensland University of Technology. SKOV-3 (steroid hormone-independent ovarian adenocarcinoma) and PC3 (steroid hormone-independent prostate adenocarcinoma) cells were kindly donated by Professor Rodney Minchin, School of Biomedical Sciences, The University of Queensland. LNCaP, PC3, DU145, OVCAR-3 and SKOV-3 cell lines were maintained in RPMI 1640 medium with 10% heat-inactivated FBS and Pen-Strep in a 5% CO_2 atmosphere at 37°C. Cells were plated at 90 µL/well of 2×10^5 cells/ml for LNCaP, DU145, OVCAR-3 and 1×10^5 cells/ml for PC3 and SKOV-3 into a flat-bottom 96-well plate. Cells were incubated for 1 h and compounds added at 10 µL/well in 10% DMSO:PBS to a final concentration of 50 and 100 µg.ml^{-1} of the compounds and 1% DMSO in the culture media in each well (n=3/compound, in 3 independent experiments). After 48 h, 10 µL MTT (5 mg.ml^{-1}) was added to each well and incubated for 4 h. Then 100 µL of acidified isopropanol (0.1 N HCl) was added to dissolve the formazan crystals and left to incubate for 30 min at 37°C. A Spectramax 250 microplate reader was used to measure the absorbance of each well at 570 nm. The percentage of cell viability for each compound was calculated by comparing absorbance with a PBS negative control. SDS was used as a positive control.

Isolation of PBMCs

The assay was undertaken with the approval from The University of Queensland Ethics Committee (Ethics Approval Number: 2009000661). A venous blood sample (4 ml) was taken from a healthy adult volunteer and PBMCs were isolated by Ficoll gradient centrifugation at 400 g for 30 min. Mononuclear cells were washed with RPMI 1640 (×3) before being resuspended in 10% FBS:RPMI. Cells were seeded at 1×10^6 cells/ml in a 96-well flat bottom plate (TPP), activated by adding 10 µg.ml^{-1} of phytohemagglutinin and incubated in a 5% CO_2 atmosphere at 37°C. After 1 h incubation, compounds were added at 10 µL/well to final concentrations of 50 and 100 µg.ml^{-1}. MTT assay was performed after 48 h incubation with the same method as described in "Tumour Cell Proliferation Assay (MTT Assay)" section.

Steroid Treatment Studies

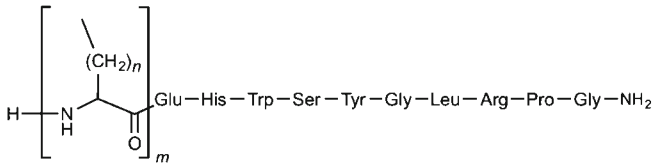
Cells were cultured to 70% confluence and then the media containing 10% normal FBS was replaced with the media containing 10% charcoal-stripped serum (CSS). After 48 h incubated cells were either seeded into 96-well plates and treated with compounds at 50 and 100 µg.ml^{-1} to perform the MTT assay or were treated with fresh CSS media containing 5 nM 17ß-estradiol or 50 nM DHT for an additional 48 h incubation. MTT assay was performed after 48 h incubation.

RESULTS AND DISCUSSION

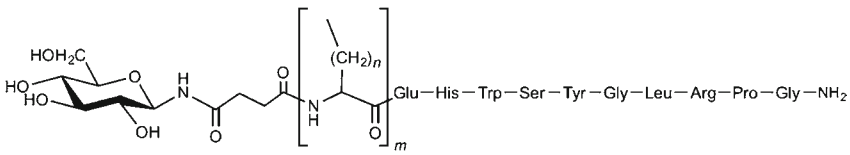
Design and Synthesis of Liposaccharide Derivatives of GnRH

GnRH peptides (**I**–**10**, Fig. 1) were synthesised by a manual solid-phase protocol with N$^{\alpha}$-Boc chemistry. In the cytosol, the N-terminal Glu residues cyclise either spontaneously or by

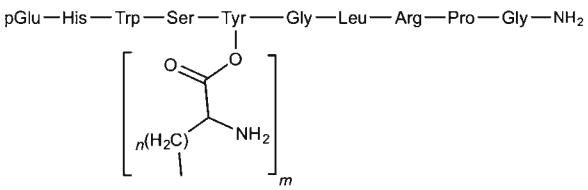
Fig. 1 The series of GnRH (glyco)lipopeptide conjugates.



Compound	*m*	*n*
1	0	0
2	1	5
3	2	5
4	1	9
5	2	9



Compound	*m*	*n*
6	1	5
7	2	5
8	1	9
9	2	9



Compound	*m*	*n*	Amino acid at position 6
10	0	0	Gly
11	1	5	Gly
12	0	0	D-Trp

enzymatic catalysis, affording the native conformation of the GnRH peptide (24). Therefore, GnRH conjugates **1**–**9** contained a Glu residue substituting for the native pyroglutamate (*p*Glu, in compounds **10**–**12**) allowing further N-terminal conjugations.

To attain a supply of exogenous GnRH with improved biological efficacy, the GnRH peptide was incorporated into a system that could enhance its uptake and stability. Lipidic adjuncts should afford enhanced transport across cellular membranes, by increasing the lipophilicity of the compound. First, lipoamino acids were synthesised from their 1-bromoalkanes using the method of Gibbons *et al.* (20). Addition of two lipoamino acids, 2-amino-D,L-octanoic acid (C8) and 2-amino-D,L-dodecanoic acid (C12), to the N-terminus in their monomeric and dimeric forms provided a good basis to determine the efficacy of different lipid conjugations (**2**–**5**). It was also proposed that the lipoamino acids would be cleaved, releasing the parent peptide, as observed previously in C12 conjugates (14). In order to address the potential problems with poor solubility, all N-terminally modified lipopeptides were conjugated at their C-terminus to a succinic acid derivative of glucose (**6**–**9**) according to the procedure outlined by Kellam et al. (25).

To expand the modification of GnRH peptide using lipoamino acids, a C12 moiety was conjugated to the hydroxyl group on the phenol group of Tyr^5, which is central to the GnRH sequence, using an *N*-hydroxysuccinimide (NHS)-activated ester on solid-phase to achieve analogue **11** (Supplementary Information, Figure S1). A manual solid-phase protocol with Fmoc chemistry was employed for the synthesis of this peptide, as Boc cleavage procedures (using hydrofluoric acid) would be too harsh for the formed labile ester bond. An acetic acid-based cleavage would have been ideal to ensure minimal hydrolysis of the ester bond, however this was not possible due to the side chain protecting groups of the GnRH sequence requiring strongly acidic conditions for cleavage. In addition, triptorelin ([w^6]GnRH, **12**), a commercially available and stabile GnRH agonist peptide, was synthesised and used as a control peptide in all cell proliferation assays.

Enzymatic Stability Profile of GnRH Conjugates

Each GnRH derivative was compared to the parent peptide to determine the effect of lipid conjugation on the stability of the peptide *in vitro*. This was achieved using a conventional colorectal adenocarcinoma (Caco-2) cell homogenate assay. It has been shown that Caco-2 cell homogenate contains various types of proteolytic enzymes found in the small intestine epithelium such as peptidases, alkaline phosphatase, and disaccharidases (26). Monitoring the stability of the compounds in the homogenate mixture was carried out using Liquid Chromatography-Mass Spectrometry (LC-MS) in a time-dependant manner (14). Each assay was carried out in quadruplicate on three separate occasions using cell passage numbers 20–45.

The unconjugated GnRH peptide (**1**) showed a half-life of 12.2 min after 2 h incubation with Caco-2 cells, which was predictably the shortest half-life observed (Table I). The lipopeptides with monomeric units of lipoamino acids increased the peptide's half-life 2-fold for compound **2** and over 3-fold for compound **4**. Interestingly, the dimeric addition of C8 in compound **3** provided no further improvement in the stability compared to the monomer form (**2**). The addition of two C12 units (**5** and **9**) resulted in significantly decreased solubility, which caused precipitation of the peptide during the assay. Therefore, half-lives of dimeric C12 compounds (**5** and **9**) could not be calculated accurately. Further addition of a glucose succinate moiety increased the half-life of both (monomeric and dimeric) C8 conjugated peptides (**6** and **7**). Analogue **7** was shown to be the most stable compound (77.4 min), followed by **6** (47.5 min). Interestingly, addition of the carbohydrate to the C12 conjugate in compound **8** conferred no significant benefit to the half-life of the non-glycosylated derivative, **4**.

The metabolic stability of similar GnRH modified compounds was analysed recently using the same assay (27,28). In one of these studies, the addition of C12 to the N-terminus of [Q]GnRH resulted in a half-life of 48.5 min, which was slightly different from the 40.7 min presented here for the monomeric C12 compound, **4** (Table I). However, there has been some evidence suggesting [Q]GnRH conjugates have longer half-lives than [E]GnRH analogues (28).

Analogue **11** had a half-life similar to that of its parent peptide (**10**). The lipoamino acid ester appeared to be completely removed from **11** by the time it was analysed by LC-MS. It should be noted that the addition of lipoamino acids resulted in a diastereomeric mixture of products due to the presence of both D and L isoforms. These epimers were previously demonstrated to have clearly different susceptibility to enzymatic degradation (17). In this assay, both epimers

Table I Stability of GnRH (Glyco)lipopeptides in Caco-2 Homogenates

	MW	$t_{1/2}$ (min)
1	1200.3	12.2 ± 0.6
2	1341.5	29.3 ± 2.2
3	1482.7	27.3 ± 2.1
4	1396.8	40.7 ± 2.9
5	1594.9	N.D.
6	1602.7	47.5 ± 3.2
7	1744.0	77.4 ± 16.5
8	1658.9	43.3 ± 5.2
9	1856.2	N.D.
10	1182.3	24.2 ± 4.3
11	1379.6	22.9 ± 4.4

were analysed in the same reaction, which resulted in a two-phase degradation pattern. Thus, half-life values were most predictive of the conformations within each mixture with the highest enzymatic susceptibility. This may explain why the half-lives of monomeric and dimeric additions of the C8 were similar, yet concentrations at the completion of the assay differed (Supplementary Information, Figure S2).

Since the selective cleavage of lipoamino acids from GnRH was observed previously (14,17), all conjugates (**1**–**11**) were screened for release of the peptide fragment by LC-MS. Three of the (glyco)lipopeptide conjugates, **2**, **4** and **8**, were observed to release GnRH (Fig. 2). C12 attached to the N-terminus of GnRH was readily cleaved from compound **4**. Compounds **2** and **8** demonstrated selective cleavage at the lipoamino acid and lipoamino acid-Glu bonds. However, compound **6** was not cleaved. It is plausible that a certain length of the alkyl chain attached to the peptide is necessary for recognition and binding to the protease. Addition of the glucose moiety in this case may have obstructed C8, but C12 was long enough to bind to the enzyme regardless (Fig. 2). Dimeric additions of lipoamino acids were also not recognised for this type of degradation, which might be due to the same chain lengths resulting in significant steric hindrance.

Apparent Permeability Across Caco-2 Cell Monolayers

An acceptable model for the absorptive phase in the intestinal epithelium is a Caco-2 cell monolayer (29). This model was therefore chosen to estimate the compounds ability to pass through the intestinal wall. Radio-labelling or LC-MS was used to quantify the amount of the compound that crossed through to the basal side of the Caco-2 cell monolayer thus providing an index for compound absorption. Apparent permeability (P_{app}) coefficients were assessed for each compound (n=4, 5) in Hank's Buffered Salt Solution (HBSS) over 2 h. ^{14}C-mannitol was used as a negative control, whereas propranolol was the positive control (Fig. 3).

The parent peptide, GnRH (**10**), displayed a P_{app} of 1.25 ($\pm$0.62)$\times 10^{-7}$ cm.s^{-1}, which was consistent with values reported elsewhere (27,30). Lipidation of GnRH with C8 (**2** and **3**) increased the permeability of GnRH more than the addition of a C12 (**4**). The differences in permeability values were explained by differences in solubility of the compounds and by variable degrees of association with the lipid membranes of the monolayers. Glycosylation of each of the lipopeptides decreased transport across the monolayers. A significant decrease (30%) in the apparent permeability of compound **3** was observed after glycosylation in compound **7** ($p<0.05$). The permeability value for **8** was not determined due to precipitation. The permeability of the ester-linked lipoamino acid conjugate, **11**, was also compared to the parent peptide **10** (Fig. 3). A 2-fold increase in the permeability of **11** was observed compared to **10** (2.4×10^{-7} cm.s^{-1}, $p<0.05$). This improvement in the permeability of **11** was higher than that of all other tested compounds when compared to the parent peptide.

During the Caco-2 permeability assay, the Glu residue in the [E]GnRH sequence (**1**) cyclised to form *p*Glu, yielding the native GnRH (**10**) in the receiver chamber (Supplementary Information, Figure S3). Glutaminyl cyclases (QCs) are known enzymes that catalyse the cyclisation of glutamic acid residues to form pyroglutamate (31). The QCs are mainly present in neuroendocrine tissues; however, mRNA expression patterns suggest that QCs are also present in Caco-2 cells (32). This cyclisation was not observed in the stability assay when using homogenates of the same cell line.

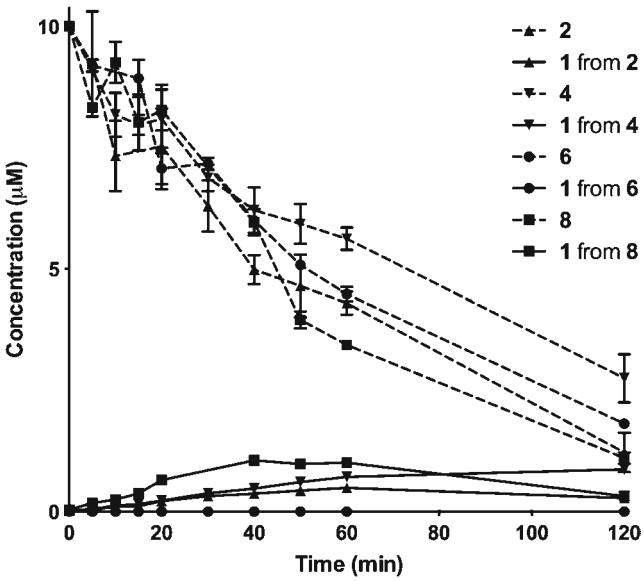


Fig. 2 Release of the parent peptide in Caco-2 cell homogenates. During incubation with Caco-2 homogenates, the parent [E]GnRH (1a, —) peptide was selectively released from the lipoamino acid-GnRH and (Glc)lipoamino acid-GnRH conjugates (– – –).

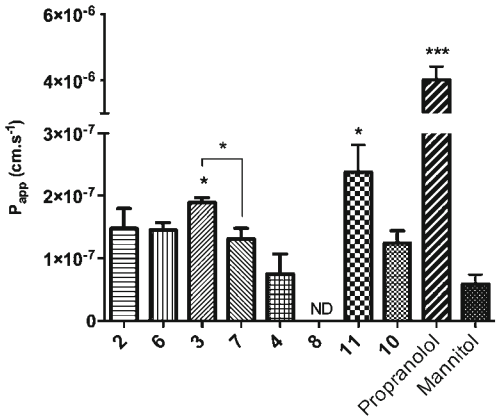


Fig. 3 Apparent permeability (P_{app}) of (glyco)lipopeptides **2**–**4**, **6**–**8** and **11** through Caco-2 cell monolayers compared to GnRH (**10**) (mean ± SEM, n = 4–5). Statistical analysis was performed using a one-way ANOVA followed by Fisher's post hoc test ($p>0.05$; *, $p<0.05$; ***, $p<0.001$).

Trans-epithelial electrical resistance (TEER) measurements were performed in HBSS buffer pre- and post- experiment as a means of assessing the integrity of the Caco-2 cell monolayers. TEER values dropped significantly after completion of the assay in all monolayers, yet they returned to normal values when incubated overnight. This observation suggests that compounds were associated strongly with junctions of the cellular membranes. A paracellular pathway was most likely utilised for the permeation across the monolayers. A significant, sustained drop in TEER values corresponds to damage to the cell monolayer. The glycosylated compounds, particularly the C8-lipoamino acid conjugates (**6** and **7**), displayed the slowest return to normal values (Supplementary Information, Figure S4).

Effect on Tumour Cell Proliferation

Cell growth, invasion and angiogenesis of peripheral tumours have been strongly linked to GnRH pathways. These effects are complex in nature and very dependent on the signal transduction pathways of particular cell types. GnRH isoforms and their synthetic relatives have been shown to have a dual action on GnRH receptor positive peripheral tissues (33). Low (nanomolar) concentrations of these peptides have exhibited a dose-dependent increase in cell proliferation, migration and invasion, yet concentrations over 100 nM have inhibited these functions (34). However, these actions may be inversed depending on the cell line and regardless of the tissue of origin. In fact, it has been shown that GnRH can both induce and inhibit invasion of the human ovarian cancer cell lines, OVCAR-3 and SKOV-3, respectively (34). The mechanisms for these differences may be explained by the expression profiles of GnRH-R and their signal transduction.

In this study, different tumour cell lines with various GnRH-R expression levels were used to investigate the effect of GnRH (glyco)lipopeptides on cell proliferation. Cells were selected from both hormone dependent (LNCaP and OVCAR-3) or hormone resistant (PC3, DU145 and SKOV-3) prostate and ovarian cancer cells; two common cancers that are treated by GnRH analogues. Furthermore, the impact of steroids was examined on the sensitivity of prostate (DU145) and ovarian (OVCAR-3) cancer cells. In these experiments, the effects of peptide analogues were investigated in a charcoal-stripped serum (CSS) media as well as in reconstituted media with either dihydrotestosterone (DHT) or 17ß-estradiol.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a colorimetric-based technique that

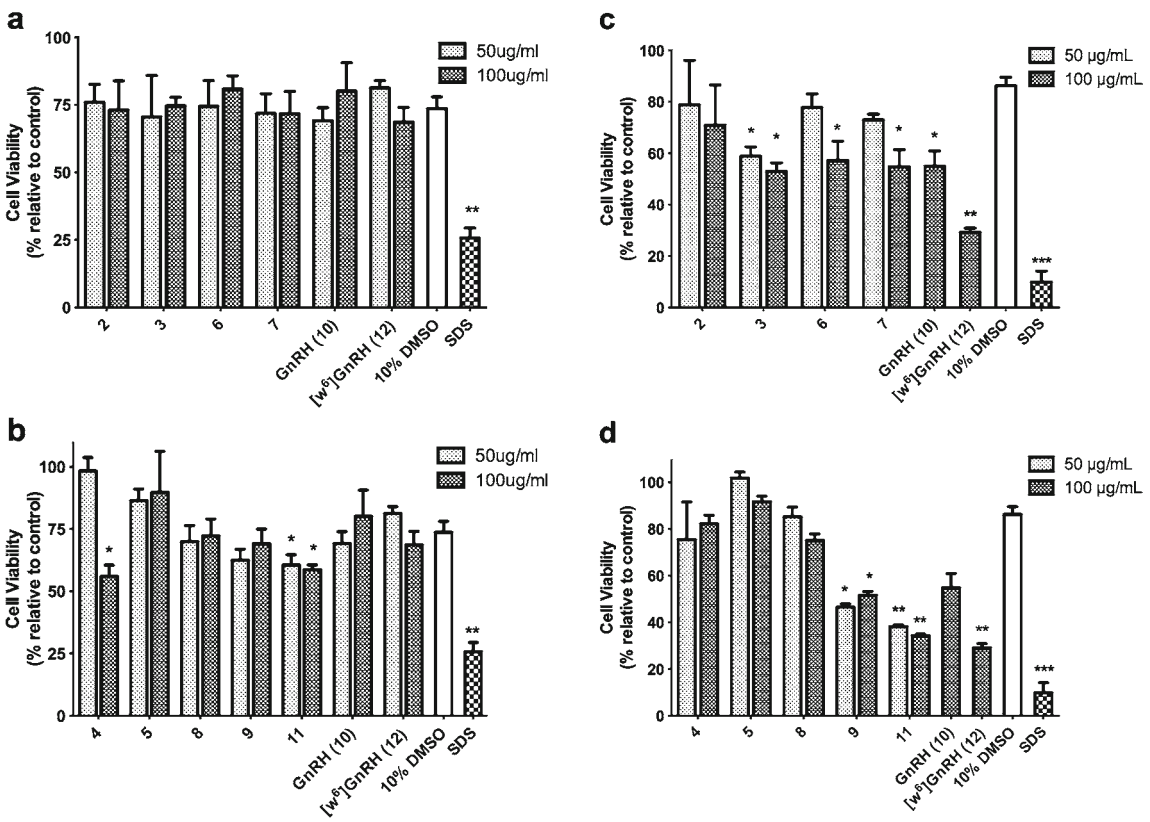


Fig. 4 Effect of C8-modified (**2**, **3**, **6** and **7**) and C12-modified (**4**, **5**, **8**, **9** and **11**) glyco(lipo) peptides on the viability of LNCaP (**a** and **b**) and DU-145 cells (**c** and **d**) as a percentage of cells treated with PBS (mean ± SEM) after 48 h incubation. Statistical analysis was performed using a one-way ANOVA followed by Dunnett's post-hoc test and comparison with the DMSO group (*, $p > 0.05$; **, $p < 0.01$, ***, $p < 0.001$).

relies on the function of mitochondrial reductase in living cells to reduce the yellow tetrazole to purple formazan crystals (35). As GnRH-R have been associated with antiproliferative effects on tumour cell lines, the MTT assay provides a method for assessing the ability of GnRH analogues to inhibit cell proliferation by signalling through these pathways. All compounds were tested in concentrations of 50 and 100 $\mu g.ml^{-1}$ by incubation for 48 h with prostatic and ovarian carcinoma cell lines – LNCaP, DU145, PC3, SKOV-3 and OVCAR-3. The surfactant, sodium dodecyl sulphate (SDS), was used as a positive control in all assays, as it is known to disrupt cell membranes and provide an indicator of assay viability. Triptorelin (**12**) has been previously shown to produce direct antiproliferative effects on different tumour cells such as ovarian (36) and prostate cancer (19) cell lines and thus was used as a positive control in this study. The buffer solution containing 10% DMSO was used as a negative control.

LNCaP cells are derived from human androgen-sensitive prostate adenocarcinoma cells and express GnRH-R (37). N-terminal C8-modified GnRH (**2**, **3**, **6** and **7**) conjugates did not produce any significant growth inhibition in this cell line (Fig. 4a). Among the N-terminally modified C12-conjugated GnRH compounds (**4**, **5**, **8** and **9**), 44% inhibition was observed in compound **4** at 100 $\mu g.ml^{-1}$. Similarly, compound **11** displayed a significant decrease in cell proliferation at both concentrations compared to the negative control (Fig. 4b, $p<0.05$). Generally, C12-conjugated analogues were more effective on LNCaP cells than C8-modified peptides.

DU145 is a human androgen-independent prostate adenocarcinoma cell line, which expresses GnRH-R (37). Compounds **3**, **6** and **7** from C8-lipoamino acid analogues and **9** and **11** from C12-modified peptides induced significant cell growth inhibition on these prostate cancer cells ($p<0.05$; Fig. 4c and d). Lipopeptide **11** was the most potent analogue with a considerably high inhibitory effect (over 60%) at 50 and 100 $\mu g.ml^{-1}$ ($p<0.01$). Due to the high growth inhibitory effect of **11**, a range of concentrations (10^{-6}-10^{-4} $\mu g.ml^{-1}$) was used and a dose–response curve obtained. The IC_{50} calculated for this compound was 26.4 ± 1.07 $\mu g.ml^{-1}$, which was comparable to triptorelin (**12**, 25.1 ± 1.14 $\mu g.ml^{-1}$). It is possible that the position of the bulky lipoamino acid residue on the GnRH backbone enhanced the conformational stability of the lipopeptide and forced its β-II formation turn for greater receptor binding (38). Dondo *et al.* showed that potent GnRH agonists that are currently available in market (goserelin and buserelin), exerted a significant and dose-dependent antiproliferative action on DU-145 cells, after 4 days of treatment (39). In another study, goserelin acetate blocked proliferation of the DU-145 cells through negative attenuation of epidermal growth factor (EGF) receptors *via* the protein kinase C pathway (40). In this study, the GnRH agonist retarded the proliferation of DU145 at 10^{-5} and 10^{-6} M. Furthermore, in a DU145 tumor xenograft model of BALB/c nu/nu mouse, a daily treatment of 50 μg/kg of goserelin significantly inhibited the tumor growth (40). This superagonist is used in the clinic to treat prostate cancer in a monthly dose of 3.6 mg following subcutaneous administration.

PC3 is a prostate cancer cell line, which expresses GnRH-R and is unresponsive to androgen stimulation (41). Neither C12- nor C8-modified (glyco)lipopeptides exerted any inhibition in the proliferation of PC3 cells (Supplementary Information, Figure S5). In contrast, analogues **2**, **4**, **5** and **8** stimulated cell growth; analogue **5** increased cell proliferation up to 42%, which was significant compared to the DMSO control ($p<0.05$). This finding was in line with a report from Ravenna *et al.* They demonstrated that PC3 cell growth was not inhibited by triptorelin, while this drug caused a significant inhibitory action on the growth of LNCaP cells at high concentrations (19).

The human ovarian carcinoma cells, OVCAR-3, express both estrogen receptor (ER) and GnRH-R (42). All C8-(glyco)lipopeptides significantly reduced OVCAR-3 cell viability at 100 $\mu g.ml^{-1}$ ($p<0.05$). OVCAR-3 cell growth was significantly inhibited by monomeric C12-modified

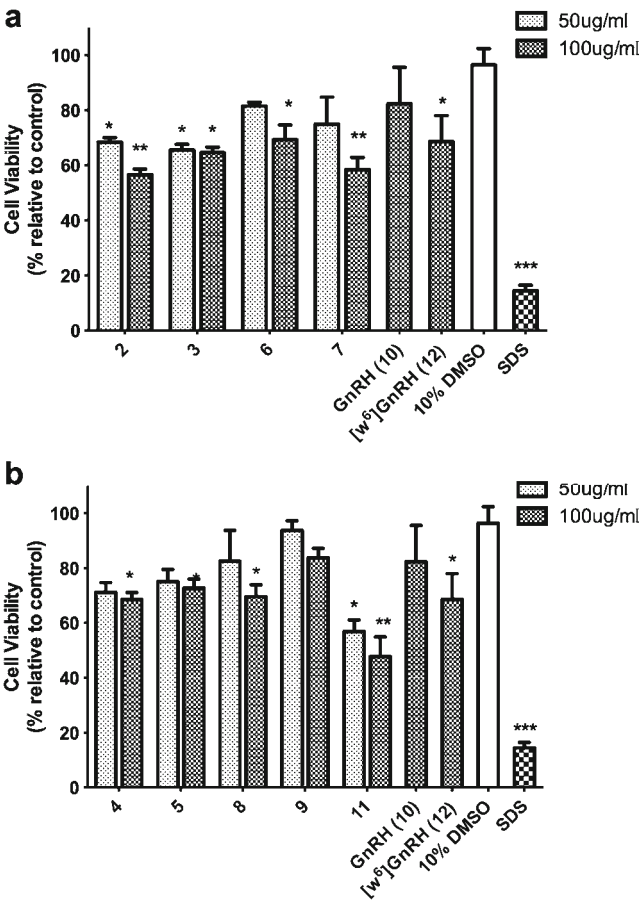


Fig. 5 Antiproliferative effects of (**a**) C8-modified (**2**, **3**, **6** and **7**) and (**b**) C12-modified (**4**, **5**, **8**, **9** and **11**) glyco(lipo)peptides in OVCAR-3 cells as a percentage of cells treated with PBS (mean ± SEM) after 48 h incubation. Statistical analysis was performed using a one-way ANOVA followed by Dunnett's post-hoc test and comparison with the DMSO group (*, $p>0.05$; **, $p<0.01$; ***, $p<0.001$).

compounds (**4**, **8** and **11**) but not dimeric C12 analogues (**5** and **9**, Fig. 5a and b). Continuous exposure of OVCAR-3 cells and xenografts to triptorelin was previously shown to produce a dose-dependent growth inhibitory effect both *in vitro* and *in vivo* (43).

SKOV-3 is an ovarian carcinoma cell line found to transcribe GnRH-II receptor mRNA, but does not express GnRH-R (44). No significant inhibitory effects of the GnRH modified compounds were observed on proliferation of SKOV-3 cells. It has been shown in some studies that the GnRH agonist, triptorelin (44), and many of the cytotoxic GnRH compounds, does not inhibit the growth of SKOV-3 cells in either *in vitro* studies or when used as xenografts in animals (45). SKOV-3 cells are known to be GnRH-R negative and estrogen-independent (46). The absence of GnRH-R could be the reason for not observing any growth inhibitory effect by the (glyco)lipopeptide analogues. However, similar to PC3 cells, compound **5** produced significant stimulatory effects on the proliferation of SKOV-3 cells ($p<0.05$; Supplementary Information, Figure S6).

It has been previously reported that the binding of GnRH analogues to their receptors inhibits the mitogenic signal transduction pathway of the EGF receptor in prostate, endometrial, ovarian, and breast cancer cell lines (47). That could explain the antiproliferative effect of GnRH analogues on GnRH-R positive cell lines (LNCaP, OVCAR-3 and DU-145) and not on GnRH-R negative cells (SKOV-3). Conversely, GnRH analogues did not inhibit the growth of GnRH-R positive PC3 cells with some compounds having stimulatory effects on this cell line. There have been contradictory data in that some GnRH-R positive cells have shown proliferative response to GnRH analogues (19). The divergence in the antiproliferative effects of GnRH analogues *in vitro* could be due to the fluctuations in levels of receptor expression during cell passage or a consequence of differences in culture conditions (11). Estrogens (42), insulin-like growth factors and members of the EGF family have been shown to impact the effect of GnRH analogues on the proliferation of cells *in vitro* (48).

The efficacy of (glyco)lipid-modified GnRH analogues was investigated in the presence or absence of steroids in OVCAR-3 and DU145 cells. In OVCAR-3 cells, removal of steroids (CSS media) affected the antiproliferative effects of compounds **5** and **8**. Depletion of steroids from media caused

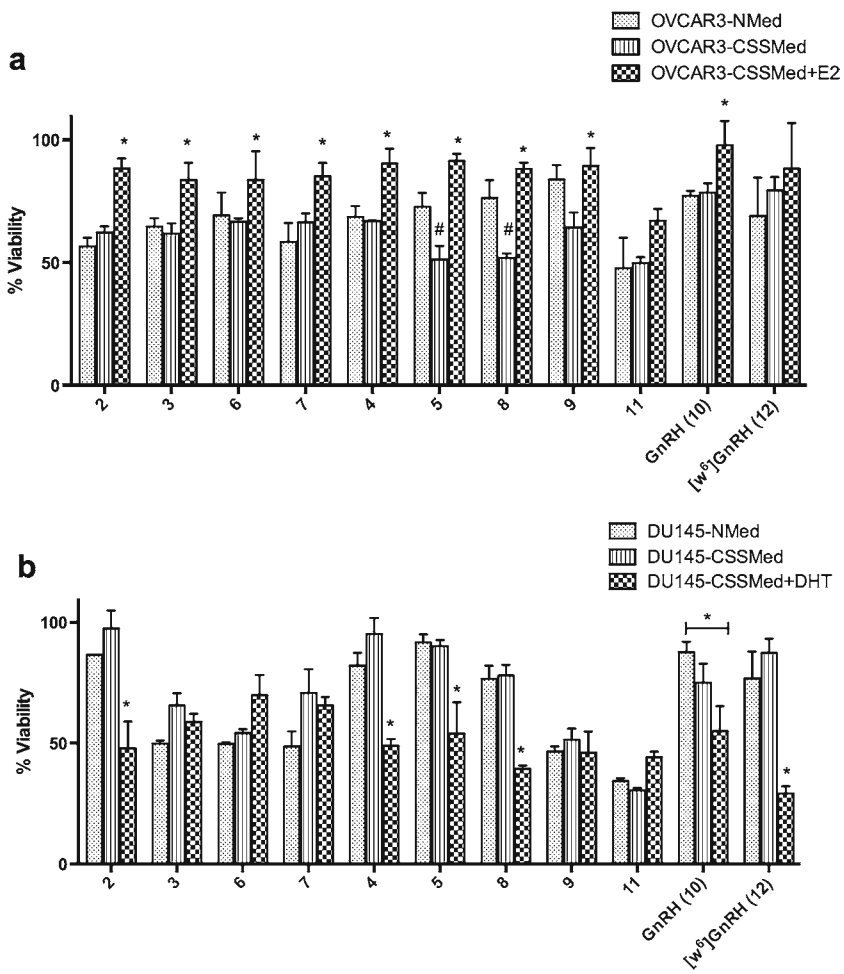


Fig. 6 Effect of the addition of (**a**) 17ß-estradiol (E2) on the sensitivity of OVCAR-3 cells, and (**b**) DHT on DU145 cells. Cells were grown in steroid free (CSS) media for 48 h then 17ß-estradiol (5 nM) or DHT (50 nM) were added to the media and incubated for 48 h. Treatment was commenced using (glyco)lipid-modified GnRH analogues at 100 $\mu g.ml^{-1}$. * $p<0.05$ CSS media treated with steroid compared to CSS media. # $p<0.05$ CSS media compared to normal media.

a 25–30% increase in the growth inhibitory effect of these compounds. Reconstitution of the media with 5 nM 17ß-estradiol decreased the sensitivity of the cells to all GnRH analogues compared to the CSS media ($p<0.05$), except for **11**. This resulted in a marked decrease in the antiproliferative activity of the compounds. Compound **11** produced significant antiproliferative activity irrespective of the presence or absence of steroids. All compounds showed better antiproliferative activity in the steroid-depleted media in the range 50–64%, with no significant difference ($p>0.05$), compared with those in 17ß-estradiol-reconstituted media. However, the efficacy of the two C12-modified compounds, **5** and **8** was shown to be more adversely affected in the presence of steroids either in normal or reconstituted media.

It has been previously shown that estrogen functions as a mitogen in OVCAR-3 cells, which can neutralise the growth inhibitory effect of GnRH-based compounds. Pre- or co-treatment with 17ß-estradiol induced a significant attenuation in the growth inhibitory effect of a GnRH agonist. This was explained by a down-regulation of GnRH-R mRNA (42).

High sensitivity of the ER positive OVCAR-3 cells to the antiproliferative effects of GnRH (glyco)lipopeptides in estrogen-depleted media is a promising finding. This could be beneficial when considering GnRH agonists for the treatment of hormone dependent cancers. In GnRH agonist therapy, there is a considerable decrease in the serum level of sex steroids. According to our results this is a favourable condition for the direct antiproliferative activity of all N-terminally modified (glyco)lipopeptides (Fig. 6).

Depletion of steroids from the culture media resulted in no effect or slight reduction in the sensitivity of DU145 cells to the (glyco)lipopeptides. However, four compounds (**2**, **4**, **5** and **8**) showed steroid-dependent growth inhibitory effects after co-treatment of the cells with DHT. These four compounds also demonstrated no significant inhibition to the growth of DU145 cells in normal media.

There are reports in the literature on the steroid-dependent antiproliferative effects of GnRH agonists (49). It has been previously shown that DHT can upregulate membrane GnRH-R expression in GnRH-R positive prostate cancer cells up to 119% (41). This receptor upregulation was observed to the same extent, when cells were treated by a GnRH agonist and DHT together. The higher sensitivity of androgen-sensitive DU145 cells to compounds **2**, **4**, **5** and **8** is plausibly due to the increased number of GnRH-R through which these compounds exert their effect. The growth inhibitory effect of the active analogues (**3**, **6**, **7**, **9** and **11**) in normal media was not significantly different when DHT was added. This suggests that although there could have been upregulation of GnRH-Rs, those analogues had reached their maximum efficacy when exposed with cells with this level of receptor expression.

Effect on the Proliferation of Peripheral Blood Mononuclear Cells (PBMCs)

A MTT assay was used to determine the toxicity of each of the compounds towards human peripheral blood mononuclear cells (PBMCs), which served as a model of non-cancerous human cells. PBMCs consist of lymphocytes, monocytes and macrophages that are isolated from blood. For this assay, PBMCs were sourced from the blood of a healthy adult and incubated with all compounds at the same concentrations tested in the tumour cell antiproliferation assays (50 and 100 µg/ml). No compounds displayed significant toxicity on PBMCs ($p>0.05$, Fig. 7). A significant increase was observed in the proliferation of PBMCs after treatment with lower concentrations (50 µg/ml) of analogue **5**. This observation was consistent with previous reports on the promotive effects of GnRH agonists on lymphocytes (50). Additionally, the immunomodulatory potential of GnRH and GnRH

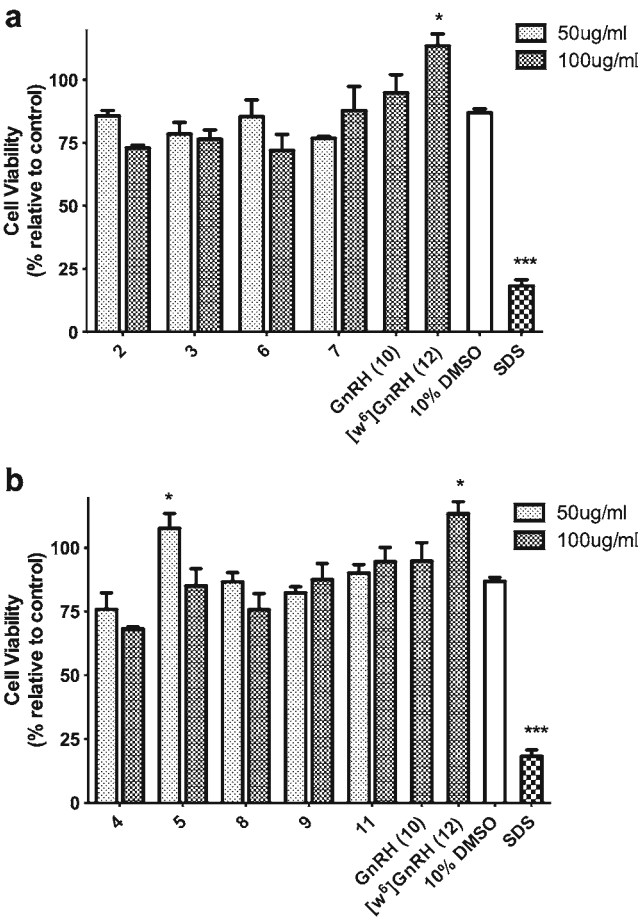


Fig. 7 Toxicity of (**a**) C8-lipoamino acid (**2**, **3**, **6** and **7**), and (**b**) C12-lipoamino acid (**4**, **5**, **8**, **9** and **11**) glyco(lipo)peptides in PBMCs as a percentage of cells treated with PBS (mean ± SEM) after 48 h incubation. Statistical analysis was performed using a one-way ANOVA followed by Dunnett's post-hoc test and comparison with the DMSO group (*, $p>0.05$; **, $p<0.01$; ***, $p<0.001$).

analogues has been reported, which has been shown to be mediated through GnRH-R (51).

CONCLUSION

The use of GnRH agonists is an important approach for the treatment of different disease states, such as gynaecological cancers. The design of an efficient method of delivery is a significant point of consideration when developing effective GnRH analogues. This study showed that lipidation and glycosylation significantly enhanced the stability of GnRH. Few of these conjugates exhibited prodrug-like characteristics that is, releasing the peptide from lipid and carbohydrate moieties. This action appeared to be dependent on the presentation of the lipid-Glu bond. Lipidation also conferred increased permeability in most cases. N-terminally modified GnRH (glyco)lipopeptides induced variable antiproliferative effects in prostate and ovarian cancer cells. Compounds produced a variable level of dependence to steroid treatment on two cancer cell lines.

The peptide with a central lipoamino acid was the most active analogue against all GnRH positive cell lines (except PC3), with an independent activity to the presence or absence of steroids (DHT and 17ß-estradiol). This is a promising compound to be further characterised in *in vivo* analysis aiming to provide a new treatment for both hormone dependent and independent reproductive cancers. This study represents another step forward in the development of GnRH-based therapeutics.

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