Effect of Lipidated Gonadotropin-Releasing Hormone Peptides on Receptor Mediated Binding and Uptake into Prostate Cancer Cells in Vitro

Rachel Stephenson PhD, Pegah Varamini PhD, Neville Butcher PhD, Rodney Minchin PhD, Istvan Toth PhD

PII: S1549-9634(14)00328-1
DOI: doi: 10.1016/j.nano.2014.06.015
Reference: NANO 969

To appear in: Nanomedicine: Nanotechnology, Biology, and Medicine

Received date: 18 March 2014
Revised date: 10 June 2014
Accepted date: 28 June 2014

Please cite this article as: Stephenson Rachel, Varamini Pegah, Butcher Neville, Minchin Rodney, Toth Istvan, Effect of Lipidated Gonadotropin-Releasing Hormone Peptides on Receptor Mediated Binding and Uptake into Prostate Cancer Cells in Vitro, Nanomedicine: Nanotechnology, Biology, and Medicine (2014), doi: 10.1016/j.nano.2014.06.015

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Effect of Lipidated Gonadotropin-Releasing Hormone Peptides on Receptor Mediated Binding and Uptake into Prostate Cancer Cells \textit{in Vitro}

Rachel Stephenson, PhD\textsuperscript{1}, Pegah Varamini, PhD\textsuperscript{1}, Neville Butcher, PhD\textsuperscript{2}, Rodney Minchin, PhD\textsuperscript{2}, and Istvan Toth, PhD\textsuperscript{1,3}

\textsuperscript{1} School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, QLD, Australia
\textsuperscript{2} School of Biomedical Sciences, The University of Queensland, St. Lucia, QLD, Australia,
\textsuperscript{3} School of Pharmacy, The University of Queensland, Woollongabba, QLD, Australia

*Corresponding Author: Professor Istvan Toth

The School of Chemistry and Molecular Biosciences
The University of Queensland
Brisbane
Australia

Phone: (617) 3346 9892
Fax: (617) 3365 4273
Email: i.toth@uq.edu.au
Short Title: Targeting prostate cancer cells with lipidated GnRH peptides

Keywords: Gonadotropin-releasing hormone, Lipoamino acid, prostate cancer, solid phase peptide synthesis, targeting

Abstract Word Count: 102

Complete Manuscript Word Count: 5156

Number of Figures: 6

Number of Tables: 3

Number of References: 44

Funding: We are grateful for funding provided by the NHMRC Program Grants (496600 and APP1037304). We also thank Thalia Guerin for her critical review of this manuscript.

Conflict of Interest: R. Stephenson, P. Varamini, N. Butcher, R. Minchin, and I. Toth declare that they have no conflict of interest.

Abstract

Gonadotropin-releasing hormone (GnRH) receptors are overexpressed on many cancer cells but not on primary cell lines. This study was designed to investigate the targeting ability and uptake of dendritic lipidated [Gln\(^1\)-GnRH peptide analogues on receptor-positive prostate cancer PC-3 cells relative to receptor-negative ovarian carcinoma SKOV-3 cells for potential application in drug delivery. Direct antiproliferative effect of these was investigated
on three GnRH-receptor positive cancer cells, PC-3, LNCaP and DU145. A significant dose dependent growth inhibitory effect was produced in DU145 cells by 5 dendrimers giving an IC$_{50}$ value of 22-35 µM. All compounds were non-toxic to the normal peripheral blood mononuclear cells.

**Keywords**

Gonadotropin-releasing hormone  
Lipoamino acid  
Prostate cancer  
Solid phase peptide synthesis  
Targeting

**Background**

Gonadotropin-releasing hormone (GnRH), also known as luteinizing hormone releasing hormone, is a decapeptide (p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly) produced in the hypothalamus. This peptide regulates the pituitary-gonadal axis, thus the reproductive system.[1-3] GnRH receptors are characteristically overexpressed in many tumours, such as breast, ovarian, pancreatic, and prostate.[1, 3-7] By contrast, the expression of GnRH receptors is not detectable in normal tissue.[1, 3-7] The elevated expression of GnRH receptors on tumour cells makes it possible to specifically target and deliver cytotoxic agents to these cells.[1, 2, 4, 8]

Prostate cancer is the most prevalent form of cancer in men and the second leading cause of death (in men) in Western countries.[5, 9] First line therapies for managing prostate cancer are localised treatments such as radiotherapy and/or prostatectomy followed by second-line
hormone-based therapies including GnRH agonists and antagonists.[1, 2, 5, 10] Recent studies have taken advantage of this differential receptor expression and used a modified GnRH peptide as a targeting moiety to enhance the relative uptake by cancer cells and decrease the relative availability to normal cells.[1, 2, 4, 8, 11] Targeting anticancer drugs to tumours may assist in improving the drugs specificity, reducing toxicity to normal cells, and reducing the possibility of multidrug resistance.[3-5]

GnRH analogues are recognised by a broad variety of GnRH-receptor positive, but not receptor negative, cells.[4, 6, 12] Using small peptides instead of conventional protein macromolecules has many advantages including ease of preparation, lower antigenicity, and increased stability. GnRH analogue-modified carriers have been used for selective delivery of diagnostic and therapeutic agents to cancer cells that overexpress the GnRH receptor.[4, 13] This ligand-targeted delivery results in increased internalisation and more effective delivery of anticancer drugs, thus overcoming the problem of multidrug resistance and toxic effects of cytotoxic drugs on normal cells.[8, 13] Extension of the GnRH C- and N-terminals by addition of lipids improves the half-life of the peptide when used in oral drug delivery applications.[14]

Natural lipidation of proteins causes a change in hydrophobicity and conformation, which promotes localisation to specific subcellular compartments.[15] In this study, lipids were included in the constructs to aid peptide self-assembly and to increase drug targeting potential.[13, 16, 17] It is known that hydrophobic modifications such as the posttranslational lipidation of proteins can result in higher membrane binding affinity, thereby affecting protein localisation and functioning.
Single lipid modification of the proteins typically allowed limited membrane affinity resulting in rapid dissociation from the membrane.[15, 18, 19] A number of thermodynamic studies have investigated the membrane binding properties of small lipitated peptides and proteins. A single unbranched lipid modification was insufficient to anchor a lipitated protein into the membrane but a second or third lipid modification provided sufficient binding energy for permanent membrane association. This lipid binding was counteracted by the polar nature of the peptide backbone and presence of charged amino acid side chains which prevented full membrane insertion.[15, 18, 19]

In the present study, lipids were assembled on a poly-lysine core which was included to enhance self-assembly properties. The peptide analogue [Gln\(^{1}\)]-GnRH (GnRH-1) was included as a targeting agent (Figure 1).

To investigate whether lipitated GnRH-1 analogues could be specifically targeted \textit{in vitro} towards GnRH-receptor positive cells, their uptake into PC-3 prostate cancer cells was compared to that of GnRH-receptor negative SKOV-3 ovarian cancer cells. Furthermore, the direct effect of these GnRH-1 analogues was examined \textit{in vitro} on the proliferation of three classic GnRH-receptor positive tumour cell lines; PC-3, LNCaP, DU145, and normal peripheral blood mononuclear cells (PBMCs) isolated from whole blood.

\textbf{Methods}

\textbf{General}

Dimethylformamide (DMF), trifluoroacetic acid (TFA), and piperidine of peptide synthesis grade were purchased from Merck Biosciences (Kilsyth, VIC, Australia). 5(6)-carboxyfluorescein (5-FAM) was purchased from Sigma Aldrich (St Louis, MO, USA).
HPLC-grade acetonitrile (MeCN) was purchased from RCI Labscan Ltd. (Bangkok, Thailand). Fmoc-protected amino acids and Rink amide MBHA resin (100–200 mesh, 0.4–0.8 mmol/g loading) were obtained from Novabiochem (Melbourne, VIC, Australia) or Mimotopes (Clayton, VIC, Australia). Preparative HPLC was carried out on a Shimadzu system equipped with a CBM-20A controller, LC-20AT pump, SIL-10A autosampler, SPD-20A UV/Vis detector set to a wavelength of 230 nm and a FRC-10A fraction collector. The analytical HPLC was a Shimadzu instrument with an LC-20AB pump, a SIL-20AHT autosampler and an SPD-M10A detector set to a wavelength of 214 nm. Electrospray ionisation mass spectrometry (ESI-MS) was performed on a PE Sciex API3000 triple quadrupole mass spectrometer, operating with a constant flow of a 1:1 mixture of solvent A (0.1% formic acid in water) and B (0.1% formic acid in MeCN/water 9:1) at a rate of 0.05 ml/min. A Zetasizer Nano ZP instrument (Malvern Instruments, UK) with DTS software was used for particle size measurements. Sizes were analysed using a non-invasive backscatter system. Measurements were taken at 25 °C with scattering angle of 173° using disposable cuvettes. PC-3, a human prostate cancer cell line and SKOV-3, a human ovarian cancer cell line, were obtained from American Type Culture Collection (Manassas, VA, USA). The LNCaP and DU145 human cell lines were kindly provided by Prof. Judith Clements at Translational Research Institute, Queensland University of Technology. Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin/streptomycin, TrypLE Express, fetal bovine serum (FBS), and Alexa Fluor 647 were obtained from Life Technologies Australia (Mulgrave, VIC, Australia). Vectorshield® mounting medium with DAPI was from Vector Laboratories, Inc., (Burlingame, CA, USA). Flow cytometry was performed on a FACSCantoTM flow cytometer (BD Biosciences, North Ryde, NSW, Australia).
Peptide synthesis

Manual Solid-phase peptide synthesis

Peptides were assembled step-wise on Rink amide MBHA resin (0.2 mmol scale) using the in situ neutralisation protocol for Fmoc solid phase peptide synthesis (SPPS).[20] N-Fmoc-protected amino acids (4.2 eq.) were activated with HATU (4 eq.) and DIPEA (5 eq.) and coupled twice for a minimum of 45 min. The following side chain protecting groups were used: Lys(Fmoc), Arg(Pbf), Tyr(tBu), Ser(tBu), Trp(Boc), His(Trt) and Gln(tBu). The Fmoc protecting group was removed by treatment with 20% piperidine in DMF for 5 min and 15 min, respectively. 2-(4, 4-Dimethyl-2, 6-Dioxocyclohex-1-ylidene) lipoamino acid [C12-LAA] was synthesised according to a previously published procedure [21] and N-protected with 1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl (Dde) [22] which was removed by treatment with 2% hydrazine hydrate in DMF (2 x 1.5 h). 5-FAM (2.5 eq.) was coupled to the resin using HOBT (2.5 eq.) and DIC (2.5 eq.) in DMF (2 x 1 h) in the dark. Additional ester-bound 5-FAM was removed by treating the resin with 20% piperidine (6 x 5 min, 6 x 15 min). Once the peptide sequence was complete, the resin was washed with DMF, MeOH and DCM, and dried under vacuum overnight. The peptide was cleaved by stirring the resin in a mixture of TFA, water and triisopropyl silane (9.5:2.5:2.5) for 3 h. Addition of cold diethyl ether precipitated the peptide, the solvent was discarded, and the peptide dissolved in a mixture of MeCN and water (1:1) containing 0.1% TFA then lyophilised.

Purification and Characterisation

Crude peptides 5 and 6 were purified by preparative RP-HPLC using a Vydac C18 column (22 mm x 250 mm) with a gradient of 100% A (water, 0.1% TFA) to 100% B (90% MeCN in water, 0.1% TFA) over 60 min at a flow rate of 20 ml/min. Crude GnRH-1. Analogues (1-4, s1-s4) were purified by preparative RP-HPLC using a Vydac C4 column (22
mm × 250 mm) with a gradient of 100% A to 100% B over 60 min at a flow rate of 20 ml/min. The collected fractions from 5 and 6 were analysed by analytical HPLC using a Vyda C18 (4.6 mm × 250 mm, 5 µm) and a Vyda C8 column (4.6 mm × 250 mm, 5 µm) with a gradient of 100% A to 100% B over 30 min at a flow rate of 1 ml/min. The fractions from 1-4, s1-s4 were analysed by analytical HPLC using a Vyda C4 column (4.6 mm × 250 mm, 5 µm) with the same gradient and solvent as listed for analogues 5 and 6. Fractions that contained pure peptide were combined and lyophilised. Yields and characterisation by HPLC and ESI-MS is shown in Table 1.

**Particle Size and Zeta Potential Measurement**

Lyophilised peptides were dispersed by vortexing in deionised water at a concentration of 1 mg/ml and leaving for 60 min prior to analysis. Measurements were performed in triplicate.

**Tissue Culture**

All cells were maintained in T-75 flasks at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. PC-3 and SKOV-3 were cultured in DMEM, LNCaP and DU145 in RPMI-1640 that was supplemented with 10% FBS, penicillin/streptomycin and 1% non-essential amino acids.

**Uptake by Flow Cytometry**

SKOV-3 and PC-3 cells were seeded into 24-well plastic plates at a density of 1.0 x 10⁵ cells per well and allowed to adhere overnight. Culture medium was removed and replaced with fresh DMEM that contained fluorescently-labelled GnRH-1 peptides at 10 µM and the cells were incubated for a further 4 h at 37 °C. Cells were washed with 1 ml PBS (3x)
and trypsinised using TrypLE Express (0.1 ml) for 5 min at 37 °C before being resuspended in 0.6 ml PBS and placed into tubes for flow cytometry. To detect the intracellular concentration of peptide, cells were treated with trypan blue (TB, 0.4%) 30 seconds prior to flow analysis to quench the surface-bound fluorescence.[23] Cells grown in the absence of GnRH-1 analogues were used as controls.

**Fluorescence Microscopy**

Cells were seeded and treated as per flow cytometry experiments. After 4 h incubation with the labelled peptide, cells were washed with 1 ml PBS (3x), treated with TB (0.4%) to quench surface bound peptide, and washed with 1 ml PBS. Cells were visualised using an Olympus IX51 inverted fluorescence microscope.

**Triptorelin Competition Assay**

SKOV-3 and PC-3 cells were seeded on coverslips in 12-well plastic plates at a density of 1.0 x 10^5 cells per well and allowed to adhere overnight. Medium was removed and replaced with 0.5 ml fresh DMEM that contained triptorelin at a concentration of 100 µM and incubated at 37 °C for 30 min. Cells were then incubated in DMEM that contained Compound 4 (1 µM and 10 µM) at 37 °C for an additional 4 h. Cells were washed with 1 ml PBS (3x) and fixed with 4% formaldehyde in cytoskeletal buffer [24] at RT for 30 min. The cells were washed with 1 ml PBS (3x). For F-actin staining, cells were incubated with Alexa Fluor 647 for 45 min at RT and mounted using Vectorshield mounting medium with DAPI. Slides were visualised using an Olympus BX61 confocal microscope.

**Calculations**
Binding index was calculated as a ratio of geometric mean fluorescence of PC-3 and SKOV-3 cells (fluorescence relative to negative control) in order to normalise fluorescence in both cell lines. Data are presented as mean ± SD. Differences between groups was determined using two-way analysis of variance (ANOVA) followed by the Tukey post hoc test and were considered statistically significant if $P$ value was <0.05.

**Isolation of peripheral blood mononuclear cells (PBMCs)**

Assay was performed with the approval from the University of Queensland Ethics Committee (Ethical Approval Number: 2009000661). A blood sample (4 mL) was collected from a healthy adult volunteer and diluted with the same volume of RPMI. Diluted blood was overlayed with 4 mL Ficoll and centrifuged at 400 g for 30 min. The white cell interface including mononuclear cells was removed and washed with RPMI 1640 (3x). Cells were resuspended in 10% FBS:RPMI and seeded at 1x10^6 cells/mL in a 96-well flat bottom plate (TPP), activated by adding 10 µg/mL of phytohemagglutinin and incubated at 37 °C in a 5% CO₂ atmosphere. After 1 h incubation, GnRH-1 compounds were added at 10 µL/well in 10% DMSO:PBS at 25 and 50 µM. MTT assay was performed after 48 h incubation with the same method as described in cell the growth assay.

**In vitro cell growth assay**

Cell proliferation was evaluated by MTT assay as published previously.[25] Briefly cells were seeded at 2.0 x 10^4 cells/well and allowed to attach for 1 h. GnRH-1 compounds were added at different concentrations (25 and 50 µM for PC-3, LNCaP and 10, 50, 100 and 200 µM for DU145 cells). After 48 h incubation, 10 µL MTT (5 mg/mL) was added to each well. Plates were incubated for a further 4 h. Formazan crystals were dissolved using 100 µL acidified isopropanol (0.1 N HCl). 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 PBS negative control. SDS was used as a positive control.

**Results**

**Peptide Synthesis and Characterisation**

The amino acid sequences of the GnRH-1 derivatives and triptorelin ([w⁶]-GnRH, a clinically available superagonist) are shown in Figure 1 and Table 1, respectively. Pure lipidic peptides were readily synthesised using step-wise Fmoc SPPS (see Supplementary Information for a detailed synthetic scheme). The lipidic moiety consisting of two synthetic lipoamino acids was synthesised as a diastereomeric mixture at the C- or N-terminus of the constructs (Figure 1). A fluorescent tag (5-FAM, Figure 1) was coupled to the N-terminus of the GnRH-1 analogues using HOBT and DIC. This step was performed in the dark to limit photo-bleaching. Removal of additional fluorophore esters was achieved by subsequent treatment of the resin with piperidine or until no further colour was observed when washed. This diastereomeric mixture resulted in broad or twin peaks in HPLC for each lipidated peptide analogue with a single peak observed for non-lipidated peptides (see Supplementary information). Following TFA cleavage, all crude compounds were purified by PR-HPLC using C18 or C4 column to >98% purity. The lyophilised peptides were obtained in 22-63% yields, characterised by ESI-MS and their purity confirmed by analytical HPLC (Table 1). Incorporation of the lipids results in a diastereomeric mixture that was not separated prior to in vitro analysis. Peptide dendrimers were assessed for their relative nanoparticular size in water to identify any significant changes between the N- and C-terminal lipidated constructs. In this study all peptides were greater than 100 nm in diameter (Table 2).
**In Vitro Evaluation**

**Cell Uptake study**

*In vitro* GnRH receptor-mediated uptake and internalisation of the GnRH-1 analogues was evaluated using GnRH-receptor over expressing PC-3 cells and low expressing SKOV-3 cells by flow cytometry and microscopy. TB was used as an extracellular quencher to distinguish between cell-associated fluorescence and fluorescence due to internalisation of peptide analogues. Optimisation of TB quenching (data not shown) was performed and found that addition of 0.4% TB 30 seconds before flow analysis gave higher quenching than addition of TB prior to the cell trypsination step.

Peptide concentration and incubation times were optimised in preliminary studies (data not shown).

Cellular internalisation of GnRH-1 analogues to PC-3 cells relative to SKOV-3 cells exhibited no significant difference for all compounds except analogues 1, 4 and 6 (Figure 2). Analogues s1-s4, containing the scrambled GnRH-1 peptide, was used as controls to show non-specific binding towards each cell line. Here, no significant difference in internalisation by PC-3 cells was observed between analogues 2 and 3 when compared to the scrambled analogues, s2 and s3. However, significant internalisation was observed between 1 and s1, and 4 and s4 (Figure 2). Interestingly, analogues 4 and 6 showed the highest binding to PC-3 cells (Figure 2 and 3). Both contained the bi-GnRH-1 peptide with 4 containing an N-terminal lipid (Figure 1).

Examination of cellular uptake using fluorescence microscopy indicated that internalisation had occurred with GnRH-1 analogues in PC-3 but not in SKOV-3 cells or with
the scrambled constructs. Figure 4 shows a representation of this for fluorescently-labelled GnRH-1 analogue 4 (has the highest cellular uptake in flow studies, Figures 2 and 3) where granules were present inside the PC-3 cells (Figure 4B) but were absent from the SKOV-3 cells (Figure 4D). Analogue 6 also showed significant uptake (Figure 2 and 3), but as the uptake was lower than 4, it was not investigated further.

Cellular uptake of analogue 4 using confocal microscopy (Figure 5) in the absence and presence of competitor triptorelin was performed. Here, the nucleus is stained with DAPI, the GnRH-1 analogue is labelled with a fluorescent tag (5-FAM), and the cell membrane stained with Alexa Fluor 647. In the absence of triptorelin, granule formation is observed in the cytosol of PC-3 cells but negligible uptake is observed when triptorelin is present. In receptor negative SKOS-3 cells, no internalisation is observed in the absence or presence of triptorelin; however, non-specific binding localised to the outer cell membrane is observed in both cell lines and is not inhibited by triptorelin treatment. Localisation of the GnRH-1 analogue appears to be isolated to the cytosol with negligible present in the nucleus.

**Tumour cell growth study**

GnRH-1 dendrimers and triptorelin were studied for their direct antiproliferative properties on three prostate cancer cell lines, PC-3, LNCaP and DU145. None of the GnRH-1 analogues, including the control peptide triptorelin, produced significant growth alteration in PC-3 and LNCaP cells at 25 and 50 µM concentrations (Figure 6a and b). However, with the exception of 5, all compounds produced significant cell growth inhibitory effects on DU145 cells in a preliminary study at 25 and 50 µM. Further experiments were performed on DU145 cells using a broader range of GnRH-1 analogue concentrations (10, 50, 100 and 200 µM) to obtain the maximum possible effect (Figure 6c), and 1, 10, 25, 50, 100 and 200 µM to
calculate the IC$_{50}$. From dose-response curves obtained for GnRH-1 analogues including triptorelin, it was shown that all compounds except 5 induced dose-dependent cell growth suppression with IC$_{50}$ values between 22 µM for analogue 1 and 35 µM for analogue 4 (Table 3). This effect of the compound was higher than that of triptorelin with an IC$_{50}$ value of 78 µM. Compound 5 produced significant reduction in the viability of DU145 cells (35%) only at 50 µM (p<0.05).

**Evaluation of GnRH-1 analogues toxicity on PBMCs**

Cell toxicity of dendrimers 1-6 was evaluated in normal PBMCs isolated from whole blood. All compounds were found to be non-toxic to PBMCs at the two concentrations used for cancer cell proliferation studies (Figure 6d). A slight increase in the proliferation (20-30%) was observed in the cells treated with compounds 2, 5 and 6 at the lower concentration, 25 µM.

**Discussion**

GnRH-1 analogues used in this study contained either two GnRH-1 epitopes organised in a dendritic manner (Figure 1; 1, 3, and 5) or two GnRH-1 epitopes presented in a linear fashion (Figure 1; 2, 4, and 6). The N-termini of analogues 3 and 4 contain C12-LAA groups compared to an internal [26] placement in analogues 1 and 2. Peptides containing no lipids (5 and 6), or a scrambled GnRH-1 analogue (s1-s4) were used as controls.

C12-LAA [14] combines essential features of amino acids and lipids and was added at different positions to investigate the potential of GnRH-1 dendrimers to target prostate cancer cells. The polar head group of C12-LAA allows for the facile inclusion into any peptide
sequence, and the solubility and hydrophobicity can be balanced by choosing an appropriate alkyl chain length.[21]

Replacement of pyroglutamic acid with glutamine was essential to enable N-terminal extension of the GnRH peptide. Glutamine is present in the endogenously synthesised peptide and under-goes spontaneous cyclisation to pyroglutamic acid, although the reaction rate of the latter is considerably higher.[14] All peptides were labelled with a fluorescent tag at their N-terminus to enable in vitro visualisation.[27]

Fusion peptides (with a visual tag) are an important tool for evaluating receptor synthesis, transport to the plasma membrane and internalisation kinetics.[28] It is unknown whether the attachment of a fluorescent tag to these constructs will alter their rate or mechanism of internalisation.[28, 29] A study which attached GFP to the C-terminus of the GnRH peptide was shown to have no significant effect on receptor-mediated internalisation kinetics compared to its radiolabelled counterpart.[28, 29] N-terminal extension by Blanchfield et al by addition of lipids to glutamic acid indicated that the GnRH-derivative was still active in oral delivery studies.[30] However, it is not known what effect addition of lipids and a fluorescent tag will have on GnRH binding and uptake of these dendritic compounds. To date, none of the amino acids in the GnRH sequence appear to be essential for receptor activation.[31]

Recently there has been an increased interest in identifying nanoparticle characteristics that are best suited to drug delivery applications. Many studies have demonstrated that nanoparticle size is a major factor affecting particle distribution into tumours. In general, nanoparticles smaller than 100 nm are considered best for tumour
targeting. However, particles sub-20 nm had rapid accumulation in tumour cells but poor retention, and particles greater than 100 nm have low tumour permeability. It has been shown that the cell surface charge of nanoparticles is also an important factor in cellular uptake.[32-34] Positively charged nanoparticles are rapidly taken up by tumour cells but can lead to significant immune reactions, hence neutral and negatively charged nanoparticles are preferable for clinical applications.[32-34] Taking into account this information and the size of the particles formed in this study (greater than 100 nm), these particles were expected to have low permeability into tumour cells, highlighting the requirement for receptor-mediated endocytosis for cellular internalisation. Additionally, N-terminally bound analogues 1 and 3 are smaller in size compared to their C-terminally bound counterparts and, as expected, the analogues with two linear epitopes (1 and 2) were larger than the analogues with single linear epitopes (3 and 4) indicating different rearrangement properties.

Cellular internalisation of GnRH-1 analogues to PC-3 cells relative to SKOV-3 cells (Figure 2) indicated the importance of the bi-GnRH-1 sequence for internalisation. Here, a negligible difference in internalisation of lipidated analogues 2, s2, 4, and s4 were observed between both cell lines (Figure 2). However, a significant difference in internalisation was observed for the non-lipidated analogue 6 when compared to PC-3 cells and the lipidated analogues (2, s2, 4, and s4). No significant difference was observed in fluorescence between mono-GnRH-1 analogues that contained lipids in either C- or N-terminal orientations when compared to the non-lipidated analogue 5 (Figure 2).

Flow cytometry (Figure 3) showed that PC-3 cells incubated with GnRH-1 analogues had higher cellular association and internalisation than SKOV-3 cells. However, significant quenching of surface-bound fluorescence by TB was observed for all analogues in both cell lines (Figure 3). Quenching studies using TB eliminated the presence of externally bound
constructs although analogues s1-s4 still exhibited a moderate to strong fluorescent signal, implying non-GnRH receptor mediated cellular association. This was supported by previous knowledge that inclusion of lipids into peptides was responsible for irreversible cell binding which would not be dissociated with extensive washing or trypsin treatment. A study investigating the extent of cellular penetration was influenced by the lipid moieties attached to the GnRH-derivative. They also found that the lipopeptides crossed the cellular cytoplasm and endoplasmic reticulum.[35, 36] The extent of binding was contributed to the increased toxicity of some compounds.[35, 36] Additionally, non-invasive cellular import of the synthetic peptide was achieved by covalent modification of a peptide chain by adding lipids.[36]

Previous studies showed that receptor-mediated internalisation of GnRH analogues, such as AN-152, could be inhibited by preincubation of the cells with triptorelin.[6, 7, 37-40] The cellular uptake of analogue 4 was examined in the presence and absence of competitor triptorelin (100x excess) in separate experiments by confocal microscopy. Non-specific binding of the GnRH-derivative, as observed in Figure 4 where fluorescently-labelled analogue 4 bound to the membrane of both SKOV-3 and PC-3 cells, precluded flow cytometry as a suitable measure of cellular uptake and inhibition. In the absence of triptorelin, uptake was seen in the form of highly fluorescent punctuate bodies in the cytoplasm of receptor positive PC-3 cells but absent in SKOV-3 cells which express low levels of GnRH receptors (Figure 5). In the presence of triptorelin minimal uptake was observed in PC-3 cells and no internalisation was seen in SKOV-3 cells (Figure 5). Non-specific binding in the periphery of the cells from both cell lines is also apparent in these images. This competition study found that uptake mediated by the GnRH-receptor was competitively inhibited by simultaneous treatment with an excess of the GnRH agonist triptorelin.[37-40]
The three classic prostate cancer cell lines, PC-3, LNCaP and DU145, which are the most widely used prostate cancer cells, were used to examine the effect of our GnRH-1 analogues on the proliferation of cells. PC-3, LNCaP, and DU145 over express GnRH receptors; however their androgen receptor status and some characteristics such as the presence and type of prostate specific antigen (PSA), p53, prostatic acid phosphatase (PAP), are different in each cell line.[41] Activation of GnRH receptors in different cell lines derived from human reproductive tissues has been shown to directly inhibit cell growth. However, contradictory data exist showing the absence of sensitivity of some cell lines to the growth inhibitory effect of GnRH analogues, which bind to the GnRH receptor.[42] This was consistent with our findings where GnRH derivatives did not affect the viability of the GnRH-receptor positive cells, PC-3 and LNCaP. LNCaP has previously been showed to elicit proliferative response to GnRH analogues.[42] Factors such as estrogen, insulin-like growth factors, or members of the epidermal growth factor family may counteract the growth inhibitory effects of GnRH analogues.[43] In contrast to the poor effect of the analogues on LNCaP and PC-3 cells, all lipid-modified compounds, as well as compound 6, produced significant dose-dependent growth suppression in DU145 cells. Analogue 5, which did not produce a dose-dependent growth inhibition, was the only compound not bearing any lipid and having only one copy of GnRH-1 in each branch. It was shown previously that combinatorial action with growth factor inhibitors is required in some cells to enhance the anti-proliferative effects of GnRH analogues. In a prior study GnRH agonist-mediated inhibition of DU145 cells was shown to be due to a decreased epidermal growth factor receptor (EGFR) level.[44] A direct antiproliferative characteristic for a GnRH receptor-mediated carrier is an added benefit to the targeted delivery of cytotoxic compounds. Conjugation of a carrier with growth inhibitory activity to an anticancer compound may reinforce the cytotoxic activity against GnRH receptor expressing tumor cells.
These studies show promising results for the use of lipidated dendritic GnRH-1 peptides as scaffolds for cytotoxic drug delivery to tumour cells by binding and internalisation via GnRH receptors. Further investigation into the mechanism of uptake in the presence of a cytotoxic drug \textit{in vitro} will be useful to establish the efficacy of this system in therapeutic applications.

In summary, we showed the facile synthesis of several fluorescent GnRH-1 analogues including lipoamino acids in different positions of the peptide sequence. Derivative 4 showed uptake in receptor positive PC-3 cells as confirmed by confocal microscopy. Absence of uptake was observed in SKOV-3 cells which express low level GnRH receptors. Non-specific binding to the cell membranes for both cell lines was confirmed using TB quenching studies with analysis by flow cytometry and microscopy. Future assays aim to investigate analogue 6 for uptake and internalisation. This investigation showed it was possible to develop fluorescently-labelled lipidated dendritic GnRH analogues, which can be selectively internalised for potential use in cell targeting applications.

Conclusion

The present study provides evidence that dendritic GnRH-1 analogues facilitated specific uptake by prostate cancer cells that overexpress the GnRH receptor. Additionally, our results showed that non-specific binding was increased when a lipid was present in the compound but did not lead to cellular internalisation. A direct growth inhibitory action of the lipidic GnRH-1 dendrimers was observed in androgen-receptor negative prostate cancer cells, DU145. These results support the premise that GnRH-1 analogues have potential value in
future drug targeting applications to treat human cancers in addition to applications in the field of vaccine development.

Acknowledgement: We thank Thalia Guerin for her critical review of this manuscript.

References


7. Volker P, Grundker C, Schmidt O, Schulz KD, Emons G: Expression of receptors for luteinizing hormone-releasing hormone in human ovarian and endometrial cancers:


Figures

Figure 1. Library of GnRH-1 analogues. GnRH-1 sequence Gln-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly for peptides 1-6 or a scrambled (s) entity, Ser-Trp-Gly-Leu-His-Pro-Gln-Gly-Try-Arg for peptides s1-s4. *5(6)-carboxyfluorescein (5-FAM).
Figure 2. Internalisation data of GnRH-1 analogues after 4 hours incubation with PC-3 and SKOV-3 cells. The intracellular fluorescence was measured after the cell surface-bound fluorescence was quenched by 0.4% trypan blue. The binding index was normalised against the negative control (Neg. Ctl., cells only) in order to normalise binding of each cell type. Negative control (Neg. Ctl.) is cells only. Each value represents mean ±SD for triplicate samples. (ns, p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001)
Figure 3. Quantitative data from flow cytometry showing the average intracellular and cell surface-bound fluorescence of FAM labelled GnRH-1 analogues after 4 h incubation with PC-3 cells (A) and SKOV-3 cells (B). Average cell-associated fluorescence was quantified by flow cytometry. Intracellular fluorescence was measured after the cell surface-bound fluorescence was quenched by 0.4% trypan blue. Cell surface-bound fluorescence is the difference between the total cell associated fluorescence and the intracellular fluorescence. Each value represents mean ±SD for triplicate samples.
Figure 4. *In vitro* cellular uptake of fluorescently-labelled analogue 4 in PC-3 (A-B) and SKOV-3 (C-D) cells. Cells were plated in 24-well plates. 5-FAM labelled peptide analogue (green) were added to the cells and incubated for 4 h at 37 °C and imaged under bright field (A, C) or fluorescence (B, D) microscopy. A-B 20x, C-D 10x.
Figure 5. *In vitro* cellular uptake of analogue 4 in SKOV-3 and PC-3 cells in the presence and absence of competitor triptorelin (100× excess). Cells were plated on culture slides. 5-FAM-labelled GnRH-1 analogues (green) were added to the cells and incubated in the presence and absence of triptorelin for 4 h at 37 °C. Membranes were stained with Alexa Fluor 647 (red). Nuclei were stained with DAPI (blue). Analysis was performed on an Olympus BX61 upright confocal microscope at 60x using oil immersion.
Figure 6. Viability of (a) PC-3, (b) LNCaP, (c) DU-145 cells and (d) PBMCs as a percentage of cells treated with PBS (mean ±SEM) after 48 hours incubation with compounds 1-6, GnRH and triptorelin ([w^6]-GnRH) in 10% DMSO. Statistical analysis was performed using a one-way ANOVA followed by the Dunnett’s post hoc test and compared to DMSO group (*, p > 0.05; ***, p < 0.001).

Tables

Table 1. Purification yields and characterisation of GnRH-1 analogues by RP-HPLC and MS

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Yield (mg (%))</th>
<th>RP-HPLC retention time (min)</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C4 Vydac Column*</td>
<td>C4 Vydac Column†</td>
</tr>
<tr>
<td>1</td>
<td>7.5 mg (35%)</td>
<td>18.18</td>
<td>24.7</td>
</tr>
<tr>
<td>s1</td>
<td>8.0 mg (40%)</td>
<td>15.2</td>
<td>15.0</td>
</tr>
<tr>
<td>2</td>
<td>8.4 mg (42%)</td>
<td>23.06</td>
<td>16.9</td>
</tr>
<tr>
<td>s2</td>
<td>5.2 mg (25%)</td>
<td>17.5</td>
<td>17.8</td>
</tr>
<tr>
<td>3</td>
<td>12.6 mg (63%)</td>
<td>23.03</td>
<td>18.0</td>
</tr>
<tr>
<td>s3</td>
<td>5.6 mg (28%)</td>
<td>19.9, 20.2</td>
<td>19.0, 20.1</td>
</tr>
<tr>
<td>4</td>
<td>5.0 mg (25%)</td>
<td>23.00</td>
<td>16.9</td>
</tr>
<tr>
<td>s4</td>
<td>20.5 mg (63%)</td>
<td>18.5</td>
<td>20.1</td>
</tr>
<tr>
<td>5</td>
<td>12.2 mg (61%)</td>
<td>15.38‡</td>
<td>15.3§</td>
</tr>
<tr>
<td>6</td>
<td>4.4 mg (22%)</td>
<td>15.30†</td>
<td>13.0§</td>
</tr>
<tr>
<td>Triptorelin</td>
<td>9.5 mg (27%)</td>
<td>18.01–§</td>
<td>18.0§</td>
</tr>
</tbody>
</table>

*MeOH gradient, † MeCN gradient, ‡ C8 Vydac column, § C18 Vydac column

Table 2. Particle size and polydispersity index (PDI) of GnRH-1 analogues measured in triplicate. Dynamic light scattering analysis was performed at 25 °C and a scattering angle of
173°. The particle suspension was diluted appropriately in distilled water before measurement.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Particle Size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>675.2 ± 34.5</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>603.6 ± 8.3</td>
<td>0.13 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>246.3 ± 0.6</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>203.2 ± 3.8</td>
<td>0.32 ± 0.02</td>
</tr>
</tbody>
</table>

Table 3. IC50 values obtained after treatment of DU145 cells with the test compounds.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Triptorelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 inDU145 cells</td>
<td>22 ± 1.02</td>
<td>26 ± 1.58</td>
<td>25 ± 1.08</td>
<td>35 ± 2.03</td>
<td>&gt;200</td>
<td>32 ± 1.75</td>
<td>78 ± 5.54</td>
</tr>
<tr>
<td>(µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>