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PEPTIDES
Peptides 26 (2005) 419–422
www.elsevier.com/locate/peptides

Importance of the central region of lamprey gonadotropin-releasing hormone III in the inhibition of breast cancer cell growth

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Received 18 August 2004; received in revised form 6 October 2004; accepted 8 October 2004
Available online 23 November 2004

Abstract

Naturally occurring isoforms of the decapeptide gonadotropin-releasing hormone (GnRH) share residues 1–4 and 9–10. lGnRH-III, the third isoform isolated in the sea lamprey has no endocrine effect in mammals but shows a direct antiproliferative effect on human breast, prostate and endometrial cancer cell lines. To investigate these features, residues 5–8 of lGnRH-III were systematically replaced with Ala. The ability of the synthetic analogs to interact with receptors on MDA-MB 231 human breast cancer cells and their effect on the growth of the same cell line were investigated.

[Ala^6]lGnRH-III and [Ala^7]lGnRH-III have neither receptor binding nor antiproliferative activity. Replacement of His^5 with Ala resulted in an analog that binds to the receptor but does not have antiproliferative activity. The results are in agreement with previous reports that modifications of Lys at position 8 are well tolerated.

Keywords: lGnRH-III; Ala-scan; Receptor binding; Cancer growth; Specific growth inhibition; MDA-MB 231

1. Introduction

Lamprey gonadotropin-releasing hormone III (lGnRH-III) is the third isoform of GnRH isolated from the sea lamprey (*Petromyzon marinus*) [19]. This decapeptide differs in residues 5–8 from human GnRH (GnRH) (Fig. 1). lGnRH-III stimulates the release of estradiol and progesterone in the adult female sea lamprey in similar concentrations to that by lGnRH-I, but has negligible endocrine activity in mammalian systems [11,19]. Yu et al. reported that lGnRH-III releases FSH selectively from rat pituitary tissue at micromolar concentrations [22], but recent studies found that lGnRH-III is not a selective FSH releasing hormone [1,10]. lGnRH-III was also shown to recognize binding sites on human cancer cells, inhibiting their growth in micromolar concentrations [11].

Analogs of GnRH are used in the treatment of estrogen-, and testosterone-dependent tumors of the reproductive tract [4–6,8,17]. A major drawback, however, is the overproduction of steroid sex hormones, the so-called “flare-up”, when agonists are used and the medical castration which follows the continuous administration of either agonists or antagonists. The mechanism of these adverse effects involves the endocrine pathway of GnRH action. The GnRH analogs also recognize a low-affinity binding site on tumor cells in vitro, but their major mechanism of action in vivo is through the

Abbreviations: lGnRH-III, lamprey gonadotropin-releasing hormone III; GnRH, gonadotropin-releasing hormone; Fmoc, 9-fluorenylmethoxycarbonyl; *t*Bu, *tert*-butyl; Trt, trytil; Boc, *tert*-butyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; HBTU, benzotriazolyl-tetramethyluronium hexafluorophosphate; TFA, trifluoroacetic acid; EDT, 1,2-ethanedithiol; RP-HPLC, reversed-phase high performance liquid chromatography; NH_4OAc, ammonium acetate; ACN, acetonitrile; ESI-MS, electron-spray ionization mass spectrometry; DMEM, Dulbecco’s modified Eagle medium; FCS, fetal calf serum; D-PBS, Dulbecco’s phosphate buffered saline; Tris–HCl, Tris-hydroxymethyl aminomethane hydrochloride; $MgCl_2$, magnesium chloride; EGTA, ethylene glycol-bis[beta-aminoethyl ether]-tetraacetic acid; DTT, dithiothreitol

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doi:10.1016/j.peptides.2004.10.007

GnRH pGlu-His-Trp-Ser- Tyr-Gly-Leu-Arg -Pro-Gly-NH_2
lGnRH-III pGlu-His-Trp-Ser- His-Asp-Trp-Lys -Pro-Gly-NH_2

Fig. 1. Primary structures of GnRH and lGnRH-III. The variable region of the GnRH family of peptides is underlined.

pituitary receptors. The first generation of GnRH antagonists used in cancer therapy had high histamine-releasing properties attributed to the close position of basic residues (D-Arg^6-Xaa^7-Arg^8) [15]. The separation of the positive charges by other residues [15] or elimination of basicity [2] reduced this side-effect but further increased the hydrophobicity of the antagonists. Analogs, such as lGnRH-III, with no endocrine activity and no tendency to cause mast cell degranulation, do not cause the above side-effects.

The bioactive conformation of sequence 5–8 of GnRH is a type II' β-turn, which was shown to be important for high affinity binding to the mammalian GnRH receptor [13,14]. Asp^6 in lGnRH-III prevents the formation of a type II′ β-turn and this peptide adopts an α-helical conformation as shown by molecular dynamics simulations and cluster analysis [21]. As a result lGnRH-III recognizes a low-affinity site on the human GnRH receptors in the pituitary.

An initial structure-activity study involving lGnRH-III has concentrated on conformational features, such as the putative salt bridge between residues Asp^6 and Lys^8 [12]. The N- and C-terminal residues were modified to enhance the stability of the peptide. Analogs were designed with focus on the variable region of the GnRH family of peptides replacing residues with amino acids having similar side chain functional groups. The role of the side chains in the variable region of the peptide has not been investigated yet. Thus, in this study a systematic replacement of residues 5–8 by Ala was performed.

2. Materials and methods

2.1. Peptide synthesis

The peptides listed in Table 1 were synthesized by solid-phase peptide synthesis on the Synergy peptide synthesizer (Applied Biosystems, Foster City, CA) using amide resin with Knorr linker [3] from Applied Biosystems and *N*-α-Fmoc protected amino acids. The following side-chain protecting groups were used: Ser-*t*Bu; His-Trt; Asp-*t*Bu and Lys-Boc. All amino acids were purchased from Novabiochem (San Diego, CA). The amino acids were coupled with a 1:1 molar mixture of HBTU/HOBt, following the protocol provided by Applied Biosystems. The peptides were cleaved from the resin and the side-chain protecting groups were removed with TFA:thioanisole:EDT:water (90:5:2.5:2.5, v/v) mixture while stirring for 15 min on ice and 2 h at room temperature. The peptides were purified by RP-HPLC on a semipreparative C18 Luna column by Phenomenex (Torrance, CA) with a flow rate of 4 ml/min. Eluent A was 50 mM NH_4OAc (pH 4.5) and eluent B was 60% ACN in 50 mM NH_4OAc. The purity of the peptides was >98%, as proven by analytical RP-HPLC on a C18 column. The peptides were characterized by amino acid analysis (Waters AccQTag) and mass spectrometry (ESI-MS).

Table 1
Binding and growth inhibition of lGnRH-III and its analogs tested on MDA-MB 231 cells or isolated membrane preparations as indicated

Analog	IC_{50} (μM)		Inhibition of cell growth[a]
	Membrane	Whole cell	
lGnRH-III	1.071 ± 1.80	0.7247 ± 0.1626	Yes
[Ala^5]lGnRH-III	11.17 ± 2.31	1.341 ± 1.70	No
[Ala^6]lGnRH-III	nb[b]	nb	No
[Ala^7]lGnRH-III	nb	nb	No
[Ala^8]lGnRH-III	15.30 ± 1.71	1.016 ± 1.53	Yes

The values are presented as mean ± S.D.
[a] Significant inhibition of cell growth at 10^{-6} M.
[b] No binding.

2.2. Radiolabeled peptides

The precursor peptide for tritiation, [$\Delta^{3,4}$-Pro^9]lGnRH-III, synthesized and purified as above, was tritiated using catalytic hydrogenation as previously described [20]. The specific activity of the peptide was 22.13 Ci/mmol.

2.3. Cell lines and culture conditions

The human breast adenocarcinoma cell line, MDA-MB 231, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were maintained in DMEM medium (GibcoBRL, Rockville, MD), supplied with 10% FCS (GibcoBRL, Rockville, MD) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.4. Cell membrane preparation

All solutions contained a cocktail of protease inhibitors formulated for mammalian cells (SIGMA, St. Louis, MO). Sub-confluent (90%) cell cultures were washed with D-PBS (GibcoBRL, Rockville, MD) and the cells were detached with non-enzymatic cell dissociation solution (Cellgro, Mediatech, Herndon, VA). Cells were washed twice in ice-cold D-PBS and incubated in hypotonic homogenization solution (5 mM Tris–HCl, 5 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, pH 7.6) for 20 min on ice followed by homogenization with a tissue grinder (Kontes Glass Co., Vineland, NJ) for 5 min on ice. The suspension was centrifuged at 985 × *g* for 15 min at 4 °C and the supernatant was collected. The homogenization step was repeated. The collected supernatant was centrifuged