The Anticancer Activity Compared Between Triptorelin and a New Gonadotropin Releasing Hormone Analogue

Mohammad Mirzaei Saleh-Abady 1, Abdolali Alizadeh 2, Fereshteh Shamsipour 3, and Hossein Naderi-Manesh 1*

1. Department of Biophysics, School of Basic Science, Tarbiat Modares University, Tehran, Iran
2. Department of Organic Chemistry, School of Basic Science, Tarbiat Modares University, Tehran, Iran
3. Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

Abstract

Gonadotropin releasing hormone (GnRH) plays a key role in reproduction. This decapeptide is synthesized and released by hypothalamus and induces the pituitary gonadotrop cells to release pituitary gonadotropin hormones. In some extrapituitary compartments GnRH and its receptor act as part of the autocrine regulatory system of cell proliferation. The anticancer activity of GnRH and its analogues has been observed by many researchers. In this study the anticancer activity of a new analogue of GnRH and triptorelin was investigated by cell proliferation assay. Results indicate that proliferation of human breast and ovarian cancer cell lines are dose-dependently inhibited. The inhibitory efficiency of the new analogue is proved to be higher than the original triptorelin. In addition to its antimitogenic activity, evidence was found for the involvement of the apoptotic mechanism in the action of the new analogue and triptorelin. In conclusion, the new analogue can be considered as a good pharmaceutical candidate.

Keywords: Anticancer activity, Breast cancer, LHRH analogue, Ovarian cancer, Peptidomimetics, Triptorelin

Introduction

Gonadotropin releasing hormone (GnRH) is the central regulator of the reproductive hormonal cascade. This decapetide is synthesized and released by hypothalamic secretory neuron which is delivered to the pituitary gland via hypophyseal portal blood system. Interaction of GnRH with its receptors on the pituitary gonadotrop cells induces the release of pituitary gonadotropin hormones, which in turn regulate gonadal steroidogenesis and gametogenesis in both sexes (1). Therefore, GnRH analogues have been used in the assisted reproduction (in vitro fertilization and embryo transfer), treatment of infertility due to polycystic ovarian diseases and fibroids. Also the efficiency of analogues of GnRH for the treatment of children with precocious puberty, endometrial carcinoma, estrogen-dependent breast cancer and prostate cancer is well established (2). There is growing evidence of autocrine/paracrine GnRH systems in human
reproductive tissues (3-6). Recent studies suggest that about 50% of breast cancer and 80% of ovarian cancer cell lines express high-affinity binding site for GnRH and its analogues as part of the autocrine regulatory system of cell proliferation. Therefore anticancer activity of GnRH analogues has been observed by many others (7-11).

Since 1972 systematic work has been preceding to synthesize agonistic and antagonistic analogues of GnRH. A powerful interest in medical applications of GnRH derivatives stimulated this undertaking. Thus, the intense activity that has occurred in this field was caused by the desire to synthesize super active analogue with prolonged biologic activity. Many agonistic and antagonistic analogues more potent than the parent hormone have been made. Several of these analogues such as triptorelin, goserelin, leuprorelin and buserelin are being used clinically and the list of their applications is steadily expanding (2).

Considerable effort has been devoted to the synthesis of peptidomimetic structures to overcome the unfavorable properties and a therapeutic deficiency of peptides (12,13). Among them is insertion of some chemical groups in the peptide sequences to increase their activity efficiency. In the present work the anticancer activity of a new GnRH analogue was investigated in comparison with triptorelin. The new analogue structure is similar to triptorelin with two extra chemical groups inserted between Leu7 and Arg8 in order to increase peptide hydrophobic properties (Figure 1). Based on previous report on the molecular mechanism of ligand interaction with the GnRH receptor (14), the hydrogen bonding and π-stacking interaction play main roles in GnRH and its receptor interaction. Therefore we expect stronger interaction between new analogue and GnRH receptor leading to greater peptide activity.

**Materials and Methods**

**Material**

[D-Trp⁶] LHRH (Triptorelin) was synthesized in our laboratory by solid phase method and the new analogue was kindly provided by Dr Balalaie et al (15). Human breast cancer cell line (T47D) and ovarian cancer cell line (OVCAR3) were obtained from Pasteur Institute of Iran. RPMI 1640 and Fetal Bovine Serum (FBS) were purchased from Gibco (USA) and Biosera (UK). Multiple 6 well-dishes were Greiner bio-one (Germany) product. Annexin V-FITC apoptosis detection kit was obtained from BD Bioscience, Pharmingen (US).

**Cell culture**

Human breast cancer cell line (T47D) and ovarian cancer cell line (OVCAR3) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated bovine serum albumin and kept at 37 °C in a humidified 5% CO₂ atmosphere.

**Cell proliferation assay**

To determine the anti-proliferation activity of the new analogue, the dose-dependent proliferation experiments were performed. In brief following steps were carried out: 2×10⁴ cells of each cell line were plated in multiple 6 well-dishes. After 24 hr, the cells were treated with 10⁻¹¹, 10⁻⁹, 10⁻⁷ and 10⁻⁵ M concentrations of each peptide. This treatment was repeated three times during six days. On the sixth day, cells were trypsinized and after Trypan-blue staining, the viable cells were counted with Neubauer-type hemocytometer and the data was expressed as the percentage of control. All proliferation assays were performed at least three times.

**Apoptotic assay**

Cytomorphological changes were observed only in treated OVCAR3 cells in 10⁻⁹ M concentration of triptorelin with an Olympus phase-contrast microscope. Cell death by apoptosis was confirmed using Annexin V-FITC apoptosis detection kit with a vital dye such as Propidium Iodide (PI) according to the manufacturer’s instructions. Briefly, OVCAR3 adherent cells that were treated with triptorelin and the new analogue were washed with the culture medium. Surface exposure of Phosphatidyl Serine (PS), as a
plasma membrane asymmetry in apoptotic cells, was detected by adding annexin V-FITC to the culture medium in a final concentration of 5 μg/ml. The cells incubated for 5 min at room temperature, then PI was added (1 μg/ml) (16,17). After rinsing with culture medium to remove excess dyes, the cells were observed by fluorescence microscope (Olympus).

**Statistical analysis**

The data from three or four dose-dependent experiments were tested by ANOVA followed by post-hoc analysis (Duncan test). All analysis performed with SPSS software.

**Results**

In T47D and OVCAR3 cell lines, proliferation was dose-dependently inhibited with different concentrations (10⁻¹¹, 10⁻⁹, 10⁻⁷ and 10⁻⁵ M) of the new analogue and triptorelin (Figures 2 and 3). The anticancer activity of the new analogue was higher than triptorelin at all concentrations. Table 1 gives a comparison of the cell number percent of cancer cell lines after treatment with triptorelin and the new analogue.

After treatment of OVCAR3 cell line with the new analogue and triptorelin, some morphologic signs of programmed cell death could be detected by invert phase contrast microscope. The vacuoles in the cytoplasm, rounding-up of cells, apoptotic bodies and bleb formation were seen as characteristic signs of the apoptotic process, while control cells had normal morphology (Figure 4). Apoptosis was also confirmed by annexin V-FITC apoptosis detection kit. OVCAR3 cells with annexinV⁻/PI⁻ were alive, annexinV⁺/PI⁻ were undergoing early apoptosis and annexinV⁺/PI⁺ were either in the end of apoptosis or were dead (Figure 5).

**Discussion**

The anticancer activity of the new analogue

| Cells, % of control | Triptorelin (M) | New analogue (M) |
|---------------------|----------------|------------------|
| T47D                | 10⁻¹¹ 10⁻⁹ 10⁻⁷ 10⁻⁵ | 10⁻¹¹ 10⁻⁹ 10⁻⁷ 10⁻⁵ |
| OVCAR3              | 10⁻¹¹ 10⁻⁹ 10⁻⁷ 10⁻⁵ | 10⁻¹¹ 10⁻⁹ 10⁻⁷ 10⁻⁵ |
| Cell lines          | 93 81 70 35 | 78 65 58 10 |
|                     | 90 66 66 33 | 83 50 17 17 |
The Anticancer Activity of Triptorelin

was higher than triptorelin at different concentrations. The improved activity of the new analogue is probably due to its stronger interaction with the GnRH receptor. Söderhäll et al reported that the insertion of hydrophobic groups in GnRH sequence would increase the π-stacking interaction of the peptide with the hydrophobic pocket of GnRH receptor and therefore improved its activity (18). Furthermore the presence of such chemical groups in the new analogue sequence is thought to increase the protease stability of the new analogue and therefore increase its biological activity.

The results of our proliferation assay correspond to similar observations obtained by other groups and are explained by the fact that GnRH and its receptor are parts of the negative autocrine regulatory system of cell proliferation (7-10). The most important features of GnRH signaling in tumors are the inhibitory interference with mitogenic pathway that results in antiproliferative actions. This peptide activates a protein tyrosine phosphatase that could inhibit the mitogenic signal transduction of growth factor receptors and therefore down regulates the cell proliferation (10). In another way our findings support previous studies that some GnRH analogues can prompt apoptosis in breast, ovarian, endometrium and prostate cancer cell lines (1,19-28). However the mechanism underlying the apoptotic effect of the analogues in the human counterpart is not fully known.

In conclusion our results show that the anticancer activity of the new analogue is more than triptorelin and it seems to be due to the known mechanisms of GnRH effect on extrapituitary compartments that was explained before. Therefore this new analogue can be considered as a good pharmaceutical candidate.

Acknowledgement

We appreciate the helpful discussion and comments given by Dr. Sadjady. The authors also express their gratitude to the Research Council of Tarbiat Modares University.
References
1. Maudsley S, Davidson L, Pawson AJ, Chan R, Lopez de Maturana R, Millar RP. Gonadotropin-Releasing Hormone (GnRH) antagonists promote proapoptotic signaling in peripheral reproductive tumor cells by activating a Gαi-coupling state of the type I GnRH receptor. Cancer Res 2004;64 (20):7533-7544.
2. Schally AV. Luteinizing hormone-releasing hormone analogs: their impact on the control of tumorigenesis. Peptides 1999;20(10):1247-1262.
3. Seeburg PH, Adelman P. Characterization of cDNA for precursor of human luteinizing hormone releasing hormone. Nature 1984;311(5987):666-668.
4. Oikawa M, Dargan C, Ny T, Hsueh AJ. Expression of gonadotropin-releasing hormone and prothymosin-alpha messenger ribonucleic acid in the ovary. Endocrinology 1990;127(5):2350-2356.
5. Palmon A, Aroya NB, Tel-Or S, Burstein Y, Fridkin M, Koch Y. The gene for the neuropeptide gonadotropin releasing hormone is expressed in the mammary gland of lactating rats. Proc Natl Acad Sci USA 1994;91(11):4994-4996.
6. Azad N, Emanuele NV, Halloran MM, Tentler J, Kelley MR. Presence of luteinizing hormone-releasing hormone (LHHR) mRNA in rat spleen lymphocytes. Endocrinology 1991;128(3):1679-1681.
7. Emmons G, Schröder B, Ortmann O, Westphalen S, Schulz K, Schally AV. High Affinity Binding and Direct Antiproliferative Effects of Luteinizing hormone-releasing hormone analogs in human endometrial cancer Cell Lines. J Clin Endocrinol Metab 1993;77(6):1458-1464.
8. Gründer C, Günthert AR, Westphalen S, Emmons G. Biology of the gonadotropin-releasing hormone system in gynecological cancers. Eur J Endocrinol 2002;146(1):1-14.
9. Völker P, Grünender C, Schmidt O, Schulz KD, Emmons G. Expression of receptors for luteinizing hormone-releasing hormone in human ovarian and endometrial cancers: frequency, autoregulation and correlation with direct antiproliferative activity of luteinizing hormone-releasing hormone analogues. Am J Obstet Gynecol 2002;186(2):171-179.
10. Emmons G, Grundner C, Gunthert AR, Westphalen S, Kavanagh J, Verschraegen C. GnRH antagonists in the treatment of gynecological and breast cancers. Endocr Relat Cancer 2003;10(2):291-299.
11. Kovacs M, Vincze B, Horvath JE, Seprodi J. Structure-activity study on the LH- and FSH-releasing and anticancer effects of gonadotropin-releasing hormone (GnRH)-III analogs. Peptides 2007;28(4):821-829.
The Anticancer Activity of Triptorelin

GnRH-II, and their receptors in humans. Endocr Rev 2005;26(2):283-306.

24. Meresman GF, Bilotas M, Buquet RA, Baranao RI, Sueldo C, Tesone M. Gonadotropin-releasing hormone agonist induces apoptosis and reduces cell proliferation in eutopic endometrial cultures from women with endometriosis. Fertil Steril 2003;80(Suppl 2):702-707.

25. Billig H, Furuta I, Hsueh AJ. Gonadotropin-releasing hormone directly induces apoptotic cell death in the rat ovary: biochemical and in situ detection of deoxyribonucleic acid fragmentation in granulosa cells. Endocrinology 1994;134: 245-252.

26. Yano T, Yano N, Matsumi H, Morita Y, Tsutsumi O, Schally AV, et al. Effect of luteinizing hormone-releasing hormone analogs on the rat ovarian follicle development. Horm Res 1997;48 (Suppl 3):35-41.

27. Mizutani T, Sugihara A, Nakamuro K, Terada N. Suppression of cell proliferation and induction of apoptosis in uterine leiomyoma by gonadotropin-releasing hormone agonist (leuprolide acetate). J Clin Endocrinol Metab 1998;83(4):1253-1255.

28. Imai A, Takagi A, Tamaya T. Gonadotropin releasing hormone analogue repairs reduced endometrial cell apoptosis in endometriosis in vitro. Am J Obstet Gynecol 2000;182(5):1142-1146.